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SECTION MEETINGS

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| **CLEVELAND** | |
| Western Reserve University | October 8, 1948 |
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### 16664 P

## Growth of Hypophysectomized Rats Injected with Growth Hormone and Fed on Different Proteins.

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A few studies [1-5] dealing with the relationship between the hypophysis and nutrition have been reported, but no systematic investigation on the nutritional requirements of hypophysectomized rats has yet been made. In this communication are recorded our preliminary results of the growth studies of hypophysectomized rats fed with proteins of different nutritive values.

*Method.* The procedure for the growth study is essentially that of Osborne and Mendel.[6] Five groups (10 animals each) of hypophysectomized rats were fed isocaloric diets containing 30% test proteins, 32% Amidex,* 24% Crisco, 2% Cod Liver Oil, 8% yeast, and 4% of a salt mixture. The vitamin supplements for each one hundred grams of the diet are:

| | mg |
|---|---|
| Thiamine HCl | 0.3 |
| Riboflavin | 0.3 |
| Niacin | 2.0 |
| Pyridoxine HCl | 0.2 |
| Calcium pantothenate | 2.0 |
| Choline chloride | 100.0 |

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[1] Lee, M. O., and Ayres, G. B., *Endocrinology*, 1936, **20**, 489.

[2] Samuels, L. T., Reinecke, R. M., and Bauman, K. L., *Endocrinology*, 1943, **33**, 87.

[3] Samuels, L. T., *Recent Progress in Hormone Research*, 1947, **1**, 147.

[4] Gordan, G. S., Bennett, L. L., Li, C. H., and Evans, H. M., Proc. Soc. Exp. Biol. and Med., 1947, **65**, 317.

[5] Gordan, G. S., Bennett, L. L., Li, C. H., and Evans, H. M., *Endocrinology*, 1948, **42**, 153.

[6] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1926, **69**, 661.

* Amidex is dextrine obtained from Corn Products Refining Co. of N.Y.

| | |
|---|---|
| Biotin | 0.01 |
| Folic acid | 0.025 |
| Inositol | 10.0 |
| *p*-Aminobenzoic acid | 25.0 |

The food consumption and the weight of the test animals were recorded twice weekly. During the entire test period of three weeks, the animals received subcutaneously 0.1 mg of a purified growth hormone preparation, isolated from beef pituitary glands, every morning.

*Results.* The data (see the accompanying table) demonstrate that animals on a protein-free diet failed to survive for even a week in spite of their average consumption of approximately 5 g per rat per day. In contrast with this the normal, unoperated rats of the same age survived for more than 2 weeks on the same protein-free diet in spite of their gradual loss of weight. The weights of animals on the test protein diets increased significantly. The increase was the smallest for soya protein diet and the greatest for the casein diet, even though the animals on soya protein consumed approximately the same amount of food as the animals on either the vitamin-free casein or lactalbumin diets. After the second and third weeks of feeding, the food consumption by rats of all groups dropped markedly. The increase in body weights was likewise less pronounced than that of the first week and could not be accentuated even when another 0.1 mg of growth hormone was injected late in the afternoon. This seems to indicate that the submaximal growth is not due to the insufficiency of growth hormone.

Our preliminary data therefore leads us to believe that soya protein, which has been demonstrated to be inferior for normal rats, did not support the growth of hypophysectomized rats as well as the proteins of higher quality, such as casein and lactalbumin. However, in this experiment the superiority of lactalbumin over casein, so often demonstrated in the maintenance of or in the promotion of the growth of normal rats, failed to reveal itself in the growth of the hypophysectomized rats.

It has been demonstrated that hyperthyroidism[7-9] in rats may increase the requirements of some of the known vitamins and perhaps some unknown nutrient factors present in liver or yeast.

Since our growth hormone preparation was most likely contaminated with thyrotropic hormone, it is possible that our observed difference in the growth promoting properties of the samples of proteins may be explained not on the basis of the amino acid content but on the contaminating nutritional factors in the test proteins. In any case, our data demonstrate that the quality of proteins in the diet can affect the growth of hypophysectomized rats, and this factor must now be emphasized in the assay of growth hormone preparations.

TABLE I.
Food Consumption and Body Weights of Hypophysectomized Rats Injected with Growth Hormone and Fed Different Proteins.

| | Weeks of feeding | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | | 1 | | 2 | | 3 | |
| Protein fed | F.C.* | Avg wt in g | F.C.* | Avg wt in g | F.C.* | Avg wt in g | F.C.* | Avg wt in g |
| Protein-free | 0 | 43.7 | 35 | 40.0 | — | — | — | — |
| Soya protein | 0 | 45.9 | 44 | 55.9 | 26 | 57.7 | 7.6 | 59.4 |
| Crude casein | 0 | 47.1 | 56 | 63.2 | 30 | 68.5 | 34 | 71.9 |
| SMA vit.-free casein | 0 | 45.5 | 47 | 61.3 | 24 | 66.3 | 25 | 67.5 |
| Lactalbumin | 0 | 44.8 | 40 | 58.9 | 22 | 64.0 | 20 | 68.8 |

* F.C. = average weight in grams of food consumed per rat per week.

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[7] Betheil, J. J., Wiebelhaus, V. D., and Lardy, H. A., *J. Nutrition,* 1947, **34**, 431.

[8] Ershoff, B. H., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 500.

[9] Robblee, A. R., Nichol, C. A., Cravens, W. W., Elvehjem, C. A., and Halpin, J. G., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 400.

16665

# Influence of Age and Mammary Development upon Mammary Carcinogenesis Following Injection of "Milk Agent."*

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It has been observed that when mice of a genetically high tumor strain but lacking the milk agent are infected with this virus after they are a few months of age they develop a much lower incidence of mammary carcinoma than if infection occurs when they are under a month of age.[1,2] Because of this observation and that of Van Gulik and Korteweg[3] that the degree of lateral bud formation in the mammary gland is determined by the presence or absence of the milk agent, it was suggested[4] that in order to be effective in the genesis of mammary cancer the milk agent virus has to be present at the time the mammary gland develops. More recently Dmochowski[5] has been able to overcome this relative "non-susceptibility" of older mice by increasing the amount of virus injected and by injecting it weekly over a 3 month period. This, together with our inability to confirm the above mentioned observations of Van Gulik and Korteweg,[6] prompted the present investigation. Since the mammae of male mice remain essentially rudimentary throughout life, the demonstration that castrate male mice bearing ovarian grafts may develop a high incidence of mammary cancer[7] suggested a suitable method for testing the relationship of the state of mammary gland development at the time of the introduction of the milk agent to mammary carcinogenesis.

*Materials and methods.* Male mice of the Zb stock (fostered $C_3H$), being genetically susceptible to the development of breast cancer but lacking the milk agent, were used for this experiment since their mammae normally show almost no development throughout life (less than those of A x Z hybrid males). Three groups of mice were employed. In the first, castration and ovarian transplantation were performed when the mice were approximately one month of age, and on the following day they were injected intraperitoneally with an extract of a spontaneous mammary tumor containing the milk agent. In the second, castration and ovarian transplantation were carried out when the animals were one month of age, but the injection of milk agent virus was postponed until the animals were 4 months of age in order to allow mammary gland development prior to the introduction of the virus. In the third group, castration and ovarian transplantation were performed when the mice were 4 months of age, and on the following day the animals were injected with a tumor extract. In the second and third groups, the animals received the milk agent when approximately the same age, but in Group 2 the mammae had developed prior to the introduction of the virus while in Group 3 mammary gland development occurred in the presence of the milk agent.

The methods of castration and ovarian transplantation were the same as described in

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* Assisted by grants from the Minnesota Division of the American Cancer Society, the Citizens Aid Society of Minneapolis, the Graduate School Cancer Research Fund of the University of Minnesota, and the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

[1] Duran-Reynals, F., and Bittner, J. J., quoted by Bittner, J. J., *Cancer Research*, 1942, **2**, 710.

[2] Andervont, H. B., Shimkin, M. B., and Bryan, W. R., *J. Nat. Cancer Inst.*, 1942, **3**, 309.

[3] van Gulik, P. J., and Korteweg, R., *Proc. Nederl. Akad. v. Wetenschappen*, 1940, **43**, 891.

[4] Bittner, J. J., *Cancer Res.*, 1942, **2**, 710.

[5] Dmochowski, L., *Brit. J. Exp. Path.*, 1945, **26**, 192.

[6] Huseby, R. A., and Bittner, J. J., *Cancer Res.*, 1946, **6**, 240.

[7] Huseby, R. A., and Bittner, J. J., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 321.

the previous paper, and ovaries of one month old Zb females were used in all instances. To obtain active virus preparations, fresh mammary tumors arising spontaneously in Z strain females were employed. The tumors were ground in a mortar with sand, extracted with distilled water, and centrifuged at relatively low speeds to remove viable cells, as well as larger cellular and connective tissue fragments. Each animal received 1 cc of a 10% extract intraperitoneally. The animals were maintained on Purina Fox Chow, and inspected weekly for the development of subcutaneous tumors. All animals developing such tumors were autopsied.

*Results.* As a preliminary study to determine the extent of glandular development at the time of the introduction of the milk agent in the second group of experimental animals, glands of Zb mice castrated and transplanted with ovaries at a month of age were studied histologically when the mice were 4 months of age. From the study of whole mount preparations it was evident that by this time several of the glands of each animal had developed extensively (all 10 glands seldom develop in male mice in response to estrogenic stimulation). The ducts of the developed glands had extended throughout the fat pads to a degree similar to that seen in virgin female mice of the same age. In addition to ductular and lateral bud development, however, there were many areas of alveolar development, a situation not encountered in virgin female mice lacking the milk agent. These areas occurred mainly near the peripheri of the glands and resembled normal alveolar lobules as seen in pregnant mice approaching parturition. Morphologically they differed from the typical precancerous lesions seen in glands of high tumor strains of mice in that the epithelium showed considerable secretory activity, and there was not the slight increase of connective tissue usually seen in precancerous hyperplastic nodules. Because of the presence of these areas of alveolar development, however, a fourth group of Zb males were castrated and transplanted with ovaries when one month of age, but they were not injected with the milk agent

TABLE I.

Summary of Tumor Data Obtained in the Various Groups of Male Mice That Were Castrated, Transplanted with Ovaries, and Injected with Milk Agent Virus at Different Ages.

| Group | Age when transplanted with ovaries, mo. | Age when injected with milk agent, mo. | No. of animals/ No. tumorous | % tumorous | | Avg age of tumor development | | Avg age of non-tumorous death, mo. |
|---|---|---|---|---|---|---|---|---|
| | | | | uncorr. | corr. | from birth, mo. | from inject. of milk agent, mo. | |
| I | 1 | 1 | 34/25 | 73.5 | 78.1 | 11.5 | 10.5 | 14.8 |
| II | 1 | 4 | 30/8 | 26.7 | 32.2 | 14.9 | 10.7 | 17.1 |
| III | 4 | 4 | 34/8 | 23.5 | 29.1 | 15.1 | 10.7 | 16.8 |
| IV | 1 | None injected | 16/0 | 0 | 0 | — | — | 20.1 |

virus in order to make certain that mammary tumors would not develop in such lobules in the absence of the virus.

The mammae of male mice castrated and transplanted with ovaries when four months of age were also studied three months after transplantation to determine if their glands had developed to the same extent as those of the younger mice. As far as could be ascertained from the inspection of whole mount preparations, this was the case.

The tumor data are summarized in Table I. In the group receiving both ovaries and milk agent at one month of age, mammary tumors developed at an earlier average age than in virgin females of the Z strain maintained in this laboratory[8] confirming the observations previously reported employing hybrid males.[7] In the 2 groups that received the milk agent at 4 months of age the incidence of mammary tumors was only about one third that seen in the group that received virus when one month of age, but there would appear to be no difference in the age at which tumors developed or in the frequency of tumor development in these latter 2 groups of animals. It is interesting that the average latent period of tumor development after the injection of the milk agent was the same in all 3 groups of animals.

Since the age distribution of tumor development after the injection of milk agent was the same for all 3 groups of mice, it would appear that the correction factor developed by Bryan and Shimkin[9] would be applicable to these data and would give some indication whether or not the lower incidence of tumors observed in the 2 groups of mice injected at 4 months of age was entirely due to the fact that these animals had a somewhat shorter time in which to develop cancer. The per cent of tumors developing during each 30 day period following the injection of the milk agent was calculated, and the mice dying non-tumorous weighted accordingly. As can be seen from the "corrected" data given in the sixth column of Table I, only a small portion of these lower incidences of tumors can be attributed to differences in the life span following the injection of the virus.

*Discussion.* The results of this experiment emphasize again that as they grow older genetically susceptible mice that lack the milk agent become less "susceptible" to the milk agent virus, at least as far as the subsequent development of mammary tumors is concerned. These data would also seem to indicate that this relative "non-susceptibility" is not due to the greater mammary gland development in female mice after one month of age since glands of 4 month old male mice that developed after the injection of milk agent had no more tumors than did glands that developed prior to the introduction of the virus.

It has been shown that young mice are more susceptible to chemical and physical carcinogens than are older mice.[10-13] As yet we have been unable to demonstrate in older mice lacking the milk agent a humoral agent capable of neutralizing active milk agent virus either *in vitro* or *in vivo*. It has been observed, furthermore, that although mice which obtain the milk agent late in life may die non-cancerous, such mice may pass the agent to their offspring which then may show a high incidence of tumors. This relative "non-susceptibility" to the development of mammary tumors following the late introduction of the milk agent may not, then, be a specific resistance to the virus but may reflect a more general increased resistance of older mice to the influence of "carcinogens." Studies are now under way in an attempt to shed more light on this possibility.

*Summary.* Employing male mice, transplanting ovaries and injecting active milk agent virus at different ages, an experiment was carried out to specifically test the relationship of the state of mammary gland development at the time of the introduction of the milk agent to mammary carcinogenesis. The results would indicate that the relative non-susceptibility of older mice is not due to greater mammary gland development at the time of virus infection.

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[8] Bittner, J. J., and Huseby, R. A., *Cancer Res.*, 1946, **6**, 235.

[9] Bryan, W. R., and Shimkin, M. B., *J. Nat. Cancer Inst.*, 1941, **1**, 807.

[10] Mider, G. B., and Morton, J. J., *Am. J. Cancer*, 1939, **37**, 355.

[11] Cowdry, E. V., and Suntzeff, V., *Yale J. Biol. and Med.*, 1944, **17**, 47.

[12] Kaplan, H. S., *Cancer Res.*, 1947, **7**, 141.

[13] Smith, W. E., and Rous, P., paper presented at the 39th Annual Meeting of the Amer. Assn. for Cancer Res., 1948.

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### 16666

## Effects of Excess Dietary *dl*-Methionine and/or *l*-Arginine on Rats.*

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The addition of low concentrations of *dl*-methionine to a casein diet increases the nitrogen balance index in the rat while the addition of higher concentrations of the amino acid decreases the index and causes a marked loss in body weight.[1,2] Loss in body weight and inhibition in growth in animals receiving excess methionine has been reported from a number of laboratories.[3-5] Excess methionine could alter the need for other amino acids, for example, by increasing the demand for them in methylating reactions,[5,6] and by promoting the formation of one type of tissue protein at the expense of others. The question is raised as to whether or not the addition of other amino acids to the diet would reduce the drain on tissue sources which excess methionine could exert. The following experiments on rats were designed to answer this question in part.

*Methods.* Nitrogen balance indexes were determined using the diet and technics previously described for the dog.[7] The diet contained 12% casein (dry weight basis). Rats were pair-fed with those fed excess methionine (4.8%) where food intake was markedly restricted. The experiments were continued for a period of 20 days with urine and fecal collections throughout the period. At the end of the period the animals were autopsied and the livers and kidneys were dried at 95° C to constant weight. The dry livers and kidneys were analyzed for nitrogen by the micro-Kjeldahl procedure and were also analyzed for total fat. The urine was analyzed for creatinine and creatine by the Folin method.[8]

*Results.* The effects of relatively large addition of *l*-arginine and/or *dl*-methionine, to casein, on the nitrogen balance index and on the excretion of creatinine in the rat are recorded in Table I.

The average nitrogen balance index for casein was 0.81. The creatinine excretion on the casein diet was 28.3 mg/day/kg body weight. The addition of *l*-arginine to casein decreased the nitrogen balance index to 0.73 so that this amount of *l*-arginine did not improve the pattern of amino acids in the diet. Addition of excess *dl*-methionine to casein decreased the nitrogen balance index markedly so that the animal was in negative nitrogen balance and increased the excretion of creatinine significantly (34.3 mg), changes which

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* These studies were supported in part by the Navy Department, Office of Naval Research.

† Gerard Swope Fellow of the General Electric Company.

[1] Brown, J. H., and Allison, J. B., Abst. 112th Meeting of the Am. Chem. Soc., 1947, 51c.

[2] Brown, J. H., Ph.D. Thesis, Rutgers University, 1948, in preparation for publication.

[3] Earle, D. P., Small, K., and Victor, J., *J. Exp. Med.*, 1942, **76**, 317.

[4] Riesen, W. H., Schweigert, B. S., and Elvehjem, C. A., *Arch. Biochem.*, 1946, **10**, 387.

[5] MacKittrick, D. S., *Arch. Biochem.*, 1947, **15**, 133.

[6] Bloch, K., and Schoenheimer, R. J., *J. Biol. Chem.*, 1941, **138**, 67.

[7] Allison, J. B., Anderson, J. A., and Seeley, R. D., *Ann. N. Y. Acad. Sci.*, 1946, **47**, 245.

[8] Folin, O., *J. Biol. Chem.*, 1914, **17**, 469.

TABLE I.
Nitrogen Balance Index and Creatinine Excretion in Rats. Average Data Obtained on 14 Animals.

| Nitrogen source | Nitrogen balance index | Creatinine, mg/day/kg B.W. | Wt loss, g |
|---|---|---|---|
| Casein 12% | 0.81 | 28.3 ± 1.2* | 17 ± 2.1 |
| 12% casein + 1.7% arginine | 0.73 | 31.2 ± 2.3 | 25 ± 5.1 |
| 12% '' + 4.8% methionine | 0.50 | 34.3 ± 0.9 | 59 ± 5.4 |
| 12% '' + 1.7% arginine + 4.8% methionine | 0.70 | 36.5 ± 0.7 | 35 ± 3.6 |

$$^{*}e = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$$

TABLE II.
Effects of Feeding *dl*-Methionine and/or *l*-Arginine in 12% Casein Diet on Amount of Liver Nitrogen and Liver Fat in Rats. Average Data Obtained on 14 Animals.

| Nitrogen source | g N/g liver* | Liver wt 100 g B.W. | g liver N/ 100 g B.W. | Liver* fat, g% |
|---|---|---|---|---|
| Control 12% casein | 0.096 ± .002 | 3.24 ± .02 | 0.096 ± .002 | 19 ± 1 |
| 12% casein + 1.7% arginine | 0.099 ± .005 | 3.03 ± .07 | 0.093 ± .002 | 18 ± 2 |
| 12% '' + 4.8% methionine | 0.112 ± .004 | 3.19 ± .06 | 0.110 ± .002 | 13 ± 1 |
| 12% '' + 4.8% '' + 1.7% arginine | 0.106 ± .001 | 2.86 ± .07 | 0.103 ± .003 | 15 ± 1 |

* Dry basis.

TABLE III.
Effect of Feeding *dl*-Methionine and/or *l*-Arginine in a 12% Casein Diet on Weight of Kidney and on Total Kidney Nitrogen in Rats.

| Nitrogen sources | $H_2O$ % | Kidney wt, g/100 g B.W. | Total nitrogen, g/100 g B.W. |
|---|---|---|---|
| 12% casein | 74 ± 1.1 | 0.69 ± 0.02 | 0.021 ± 0.002 |
| 12% '' + 1.7% arginine | 76 ± 1.2 | 0.67 ± 0.02 | 0.019 ± 0.002 |
| 12% '' + 4.8% methionine | 76 ± 1.2 | 0.95 ± 0.04 | 0.027 ± 0.003 |
| 12% '' + 1.7% arginine + 4.8% methionine | 74 ± 1.1 | 0.79 ± 0.05 | 0.023 ± 0.002 |

are associated with loss in nitrogen stores. The addition of arginine to the casein-methionine mixture increased the nitrogen balance index (0.7) above that for *dl*-methionine and casein alone, the creatinine excretion remaining at a high level (36.5 mg/day/100 g body weight). The increase in excretion of creatinine associated with excess methionine and arginine may be the result of increased synthesis of creatine.[6]

The close correlation between nitrogen balance index and weight loss is illustrated also in Table I. These data demonstrate that the loss in weight was much higher in animals fed the excess methionine and that the presence of arginine with the excess *dl*-methionine prevented some of this loss.

The effects of feeding *dl*-methionine and/or *l*-arginine in the 12% casein diet on the amount of nitrogen in the liver and on liver fat are recorded in Table II.

The addition of *l*-arginine had little effect on the nitrogen of the liver but *dl*-methionine increased it, an increase which took place in the presence of a reduction in retention of nitrogen and a loss in body weight. The marked lipotropic effect of methionine is also shown by the reduction in the per cent of liver fat. Indeed the body fat is generally reduced below that found in the control animals, a reduction which contributed to the loss in body weight.

The addition of *dl*-methionine to the casein diet increased the size of the kidney while *l*-arginine alone had no effect (See Table III). The addition of *l*-arginine plus *dl*-methionine tended to increase the size of the kidneys but the increase was by no means as marked as with *dl*-methionine alone, arginine having an antagonistic effect to methionine.

*Summary.* When excess *l*-arginine is added to casein, the nitrogen balance index is reduced slightly. An excess of *dl*-methionine on the other hand reduces the nitrogen balance index markedly and the excretion of creatinine is increased. When both *l*-arginine and *dl*-methionine are added to casein the nitrogen balance index is not reduced as much as it is in the presence of casein plus methionine, but the creatinine excretion is increased. The animals receiving 12% casein and pair-fed with the group receiving 12% casein plus 4.8% *dl*-methionine lose weight slightly over a 20-day period. Those receiving 12% casein plus 4.8% methionine lose weight markedly, a loss which is overcome in part by adding 1.7% *l*-arginine to the methionine diet. The decrease in weight in the presence of a large excess of methionine is associated with a loss in body nitrogen and fat. The addition of *dl*-methionine increases the amount of nitrogen in the liver and reduces the amount of fat. Feeding *dl*-methionine plus casein increases the size of the kidney, an effect which is antagonized by *l*-arginine.

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## 16667

# Influence of Protein Reserves on Nephrectomized and Renal Artery Ligated Dogs.[*]

E. MYLON AND P. GOLDSTEIN.[†] (Introduced by M. C. Winternitz)

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The contrasting symptomatology, speed of elevation of blood nonprotein nitrogen and survival period of animals, after bilateral ligation of the renal arteries and removal of the kidneys has been reported.[1]

Ligation of the arteries results in earlier and more severe evidence of toxicity, more rapid rise in blood nonprotein nitrogen and is more rapidly fatal. It is further of interest that the intravenous injection of renal extracts after bilateral nephrectomy tends to reproduce the effects of ligation of the arteries. In these circumstances toxicity and elevation of blood nonprotein nitrogen develop earlier and the duration of life is materially shortened. This evidence of association of accelerated nitrogen catabolism and toxicity is reinforced by the results of protein feeding to animals after removal of the kidneys[2] and also after substantial reduction of kidney function.[2,3] It points to a probable pathological nitrogen metabolism with production of toxic intermediates that represent some part of the augmented blood nonprotein nitrogen. Two factors seem to be concerned in the more rapid manifestations of toxicity and the rise in blood nonprotein nitrogen. They involve on the one hand some non-excretory renal function not yet known and on the other availability of tissue protein. The latter can be reasonably controlled by diet; this hypothesis is the basis of the experiments that follow.

*Materials and Methods.* Two groups of animals were given protein poor and protein rich diets respectively for protracted periods prior to the ligation of the renal arteries or the removal of the kidneys.

The daily ration of the protein poor diet per kilogram dog consisted of: Farina 15.0 g, Karo 2.5 g, Cod Liver Oil 0.4 g, Fresh yeast

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[*] Aided by a grant from the Commonwealth Fund.

[†] James Hudson Brown Memorial Junior Fellow.

[1] Winternitz, M. C., Mylon, E., Waters, L. L., and Katzenstein, R., *Yale J. Biol. and Med.*, 1940, **12**, 623.

[2] Mylon, E., Smith, E. R., and Goldstein, P., *Am. J. Physiol.*, 1948, **153**, 55.

[3] Addis, T., *Tr. Assn. Am. Phys.*, 1940, **55**, 223.

cake 0.2 g, Salt mixture 0.3 g. The diet corresponds to about 80 cal. per kilogram dog.

The composition of the salt mixture follows:

| | g |
|---|---|
| Potassium chloride | 30 |
| Sodium chloride | 40 |
| Magnesium chloride | 20 |
| Di-sodium phosphate | 100 |
| Monocalcium phosphate | 100 |
| Calcium lactate | 40 |
| Ferric ammonium citrate | 10 |

Nine animals included in this group were maintained on this diet for from 50-60 days. Then the kidneys were removed in 3 and the renal arteries ligated in the remaining 6.

Another group of 13 dogs were allowed 75 g each day of freshly ground horse meat per kilo—approximately the same caloric value as that of the protein poor diet. After 14 days of such feeding the renal arteries of seven of these animals were ligated and the kidneys removed in the other 6.

All operative procedures were facilitated with nembutal anesthesia, 30 mg per kilo. The exposures of the kidneys were by bilateral flank incisions and care was taken in ligation of the arteries to avoid inclusion of vein or ureter. The nonprotein nitrogen of the blood was determined with the half-micro Kjeldahl method.

*Results and Discussion.* As can be seen from Table I and Fig. 1 and 2, the height of the nitrogen catabolism as well as the degree of toxic symptoms following either operative procedure, are greatly influenced by the diet fed prior to the operations. Table I demonstrates both elevated nitrogen catabolism and reduction of the survival period of meat fed animals (Group 1 and Group 3) as compared to the 2 groups of dogs conditioned by the protein poor diet (Group 2 and 4).

In addition, the figures obtained for the animals of Group 1 and 3 indicate anew the contrasting symptomatology between bilateral ligation of renal arteries and removal of the kidneys. It can be seen that the former procedure results in a more rapid rise in blood

TABLE I.

Average N.P.N.'s (in mg %) Following Bilateral Renal Artery Ligation.

| | | | Days | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Group | Diet | No. of dogs | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| 1 | meat | 7 | 45.5 | 115 | 211 | 293* | | | | | |
| 2 | carbohydrate | 6 | 27 | 61.9 | 92.1 | 130.9 | 181.9 | 209.9† | 283‡ | 363‡ | 395‡ |

* Avg of 3 survivors.
† ,, ,, 4 ,,
‡ Values of the 6th dog.

Average N.P.N.'s (in mg%) Following Bilateral Nephrectomy.

| | | | Days | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Group | Diet | No. of dogs | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| 3 | meat | 6 | 47.9 | 91.9 | 150.2 | 217.3 | 278 | 307* | 310† | | | | | |
| 4 | carbohydrate | 3 | 30 | 56.8 | 87.1 | 120.6 | 163 | 190.3 | 234.7 | 281.6‡ | 220§ | 208 | 252 | 327 |

* Avg of 3 survivors.
† ,, ,, 2 ,,
‡ Avg of 2 survivors.
§ Values of the third dog.

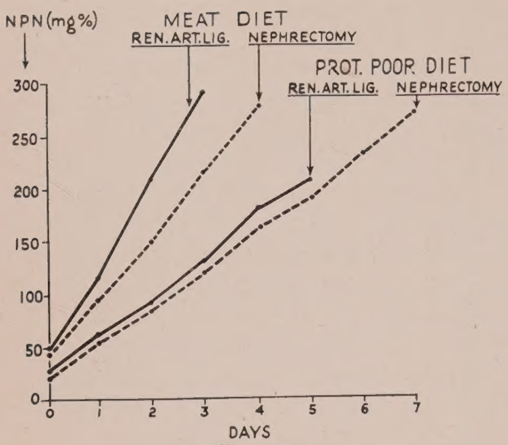

FIG. 1.

Nonprotein nitrogen of the blood of renal artery ligated and nephrectomized dogs pre-fed either a meat or a carbohydrate diet.

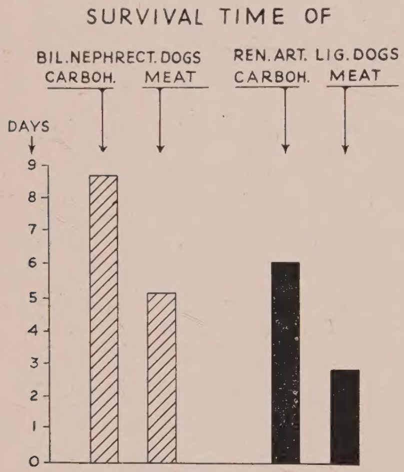

FIG. 2.

Survival time of renal artery ligated and nephrectomized dogs pre-fed either a meat or a carbohydrate diet.

nonprotein nitrogen[‡] and in a significant reduction in the survival time. While obviously these effects are caused by substances liberated from the anoxemic kidney the mechanism of their action is not yet known. One lead may be available in the fact that these derivatives were only slightly active in animals of group 2, *i.e.* in those greatly deprived of their protein reserves. This observation suggests that renal substances, in themselves not very toxic, stimulate the intracellular nitrogen catabolism and that this stimulation is associated with the formation of toxic protein intermediates.

*Summary and Conclusions.* Renal artery ligation as well as nephrectomy was carried out in 2 groups of dogs, one maintained prior to the operation on a carbohydrate, the other on a protein diet. Following either operative procedure the nitrogen catabolism was more rapid, the toxic symptoms more severe, and the survival time shorter in protein pre-fed animals as compared with the carbohydrate group. In the former the contrasting results of renal artery ligation and nephrectomy are marked. It may be concluded both that substances derived from the ischemic kidney stimulate catabolism of protein reserves and that the toxic symptoms following renal artery ligation are associated with accelerated nitrogen catabolism.

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[‡] The differences in the nonprotein nitrogen between animals of Groups 1 and 3 for 48 hours and 72 hours after the operative procedures are highly significant. The $p$ values for these two time periods are $<0.01$.

16668

# Antibacterial Activity of Gentisyl Alcohol and Homogentisic Acid.

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(Introduced by J. C. Forbes)

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It has been reported recently that gentisyl alcohol[1] and numerous derivatives of gentisyl alcohol[2,3] have practically the same antibacterial activity as patulin* against *S. aureus* in plate tests. Raistrick and coworkers[5] had reported previously that gentisyl alcohol had only slight activity against *S. aureus*, in contrast to the strong antibacterial activity of patulin.[6]

In preparation for studies involving homologous alcohols related to gentisyl alcohol we prepared gentisyl alcohol in order to test its antibacterial activity against *S. aureus*. In contrast to the reported findings of the Swiss workers,[1-3] but in agreement with the original report of Raistrick and coworkers,[5] our results with gentisyl alcohol in the cylinder plate penicillin assay technic indicated only slight antibacterial activity over a broad pH range. For this reason work with the higher homologues of gentisyl alcohol is not contemplated at the present time, but in view of the relationship of homogentisic acid to the next higher homologue of gentisyl alcohol we have also tested this compound for antibacterial activity against *S. aureus*. We found it to have definite but slight antibacterial activity of about the same order of magnitude as that of gentisyl alcohol. Curves showing the antibacterial action of gentisyl alcohol, homogentisic acid and penicillin in relation to pH of the medium are presented in Fig. 1.

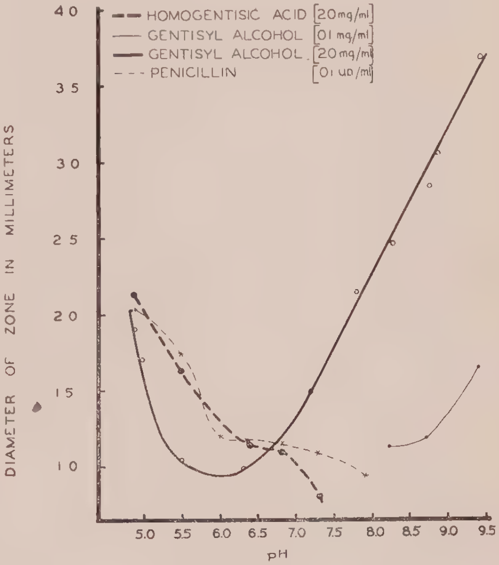

FIG. 1.

Effect of pH of the medium on the antibacterial activity of penicillin, gentisyl alcohol, and homogentisic acid.

*Experimental.* Gentisyl alcohol was prepared by catalytic hydrogenation of gentisaldehyde according to the method of Birkinshaw, Bracken and Raistrick.[5] Synthetic homogentisic acid was prepared by a procedure which we intend to present in detail elsewhere. These compounds were purified by recrystallization several times and gave melting points agreeing with those reported in the literature.

Essentially the same cylinder plate procedure as that reported by Schmidt and Moyer[7] for the assay of penicillin was used to determine antibacterial activity of the compounds. The only variation from this technic was in use of *Difco "penassay"* seed agar adjusted to variable hydrogen ion concentrations and the use of distilled water instead of buffer solutions to dissolve the penicillin and test compounds. Cylinders 8 mm in diameter were used, and *S. aureus* 209P was the test organism. All tests were run in quadruplicate, and an average of the 4 zone diameters was taken for the final reading.

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[1] Brack, A., *Helv. Chim. Acta*, 1947, **30**, 1.

[2] Renz, J., *ibid.*, 1947, **30**, 124.

[3] Seebeck, E., *ibid.*, 1947, **30**, 149.

* Also known as clavacin, clavatin, claviformin, and expansin.[4]

[4] Waksman, S. A., *Microbial Antagonisms and Antibiotic Substances*, 2nd Ed., The Commonwealth Fund, New York, 1947, p. 210.

[5] Birkinshaw, J. H., Bracken, A., and Raistrick, H., *Biochem. J.*, 1943, **37**, 726.

[6] Birkinshaw, J. H., Bracken, A., Michael, S. E., and Raistrick, H., *Lancet*, 1943, **2**, 625.

[7] Schmidt, W. H., and Moyer, A. J., *J. Bact.*, 1944, **47**, 199.

*Discussion.* A concentration of 20 mg/ml of gentisyl alcohol or of homogentisic acid was necessary to produce antibacterial activity comparable to that shown by 0.1 unit/ml of the working standard of penicillin. Brack[1] reported antibacterial activity with concentrations of gentisyl alcohol of 100 γ/cc over the pH range 5.0 to 8.5. This degree of antibacterial activity was not observed in our experiments. We did note slight activity with this concentration of gentisyl alcohol in the pH range above 8.2. With 2% gentisyl alcohol antibacterial activity was more marked at low and at high pH values. Some oxidation of the compound occurs in the assay procedure, especially in alkaline media, and is evidenced by browning of the zones of inhibition. The possibility that oxidation products might have contributed to the antibacterial activity of gentisyl alcohol noted in these cases has not been investigated. It will be noted that the pH activity curve usually observed with penicillin was obtained for control and comparative purposes in these experiments.

The various results of different laboratories might possibly be due to differences in media, test organism or to a combination of these two important factors, and they suggest that these factors may be critical in the evaluation of the antibacterial activity of gentisyl alcohol or its derivatives.

The slight antibacterial activity exhibited by homogentisic acid has not to our knowledge been described previously. This activity decreased rapidly with increase in the pH of the assay agar.

*Summary.* Gentisyl alcohol in our experiments was found to have only slight antibacterial activity over a broad pH range. Our results with this compound are at variance with those of Brack[1] but are in agreement with the original report of Raistrick and coworkers.[5]

Homogentisic acid was found to have a definite but slight antibacterial activity.

The effect of pH of the medium on the antibacterial activity of these compounds against *S. aureus* was studied.

### 16669

## Purification of Psittacosis Virus by Alcohol Precipitation and Centrifugation.

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(Introduced by Keith H. Lewis)

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A number of procedures have been described in which chemical agents such as calcium phosphate,[1] alum,[2] protamine,[3] zinc and magnesium hydroxide[4] are utilized in the concentration and partial purification of influenza virus. Recent experiments were reported by Cox *et al.*[5] on the treatment of this virus with organic solvents such as methyl and ethyl alcohols and the possibility of applying the same procedure in the purification of other viruses was suggested. Accordingly, experiments were conducted to determine (1) the effects of methyl and ethyl alcohol on the viability of psittacosis virus and (2) the purification value of this method compared to simple centrifugation.

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[1] Stanley, W. M., *Science,* 1945, **101**, 332.

[2] Bodily, H. L., Corey, M., and Eaton, M. D., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 165.

[3] Chambers, L. A., and Henle, W., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 481.

*Materials and Methods.* The 6BC strain of virus used in these tests was originally obtained from Dr. K. F. Meyer. Following inoculation with an allantoic passage strain of this virus, the eggs were incubated at 37°C for 4 to 5 days. They were then candled and living embryos chilled at 4°C for 12 to 18 hours, after which the allantoic fluid was harvested aseptically and used as starting material. Usually the harvest was held another 18 to 24 hours at 4°C pending the results of sterility tests.

Infectivity titrations were performed by the single dilution method, *i.e.*, the inoculation of 0.25 ml of a single dilution of each test aliquot into the yolk sacs of 30 embryonated eggs. The eggs were candled at 24 hour intervals thereafter. The $LD_{50}$ is then calculated from the average day of death of the group of embryos.[6]

Total nitrogen determinations were made by the micro-Kjeldahl method.*

The alcohol precipitation method used was comparable to that recently described by Cox *et al.*[5] To aliquots of infected allantoic fluid, cooled to 0°C, absolute methanol or ethanol (previously chilled to –40°C or below) was added dropwise through a capillary pipette until final concentrations of from 5% to 40% by volume were reached. The mixtures were held at 0°C for approximately 3 hours and then centrifuged for 30 minutes at 3500 rpm in a No. PR-1 International Refrigerated centrifuge (0°C) equipped with an angle head rotor. The supernatant fluid was removed with a syringe and discarded. The precipitate was resuspended to original volume in 0.1 molar phosphate buffer, pH 7.4, and gently agitated on a mechanical shaker for 1 hour at room temperature. The suspension was then centrifuged at 1500 rpm for 15 minutes at room temperature in a No. I International centrifuge equipped with a horizontal rotor. The supernatant fluid, removed from the insoluble sediment, was titrated for virus activity in eggs by the method described. No pH adjustments were made.

*Results.* Following the completion of preliminary experiments on the behavior of psittacosis virus to the alcohol precipitation technic, tests were conducted to determine the effect of varying concentrations of methanol and ethanol on the viability of the virus.

*Precipitation of allantoic fluid virus with varying concentrations of absolute methanol.* To aliquots of infected allantoic fluid, previously centrifuged at 1500 rpm for 15 minutes to remove supposedly only large particles of extraneous material, absolute methanol (–40°C) was added in concentrations of from 5% to 40%, and the samples treated according to the procedure outlined. A control aliquot of virus from each starting virus suspension was held at 0°C during the period of the extraction. Upon completion of the process, each alcohol fraction was titrated along with the control for virus infectivity in embryonated eggs.

The results shown in Table I are typical of the observations made on several repeat experiments. There did not appear to be an optimum concentration of methanol between 5% and 30% which would consistently yield high recovery values of living virus as measured by infectivity tests. Although the results of tests made for mg total N/ml indicate that in most instances better than 90% of the nitrogen had been removed, the loss in infectivity titer varied from 0.2 to

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[4] McKinstry, D. W., and Cox, H. R., 1945, Unpublished experiments.

[5] Cox, H. R., Van Der Scheer, J., Aiston, S., and Bohnel, E., *J. Immunol.*, 1947, **56**, 149.

[6] Golub, O. J., *J. Immunol.*, 1948, in press.

*Determinations made by Dr. Benjamin Warshowsky of the Biological Division, Camp Detrick, Frederick, Md.

TABLE I.
Precipitation of Psittacosis Virus from Allantoic Fluid with Varying Concentrations of Methanol.

| | | | Precipitation by absolute methanol concentration of: | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Lot No. | Type of data | Control | 5% | 10% | 15% | 20% | 25% | 30% | 40% |
| 1 | $LD_{50}$ (neg. log) | 6.2 | 6.0 | 5.8 | 5.9 | | | | <2.0 |
| | % recovery | | 63 | 40 | 50 | | | | <0.1 |
| | Mg N/ml | 0.516 | 0.017 | 0.018 | 0.019 | | | | 0.057 |
| 2 | $LD_{50}$ (neg. log) | 7.0 | | | 6.6 | 6.5 | 6.8 | 6.5 | <2.0 |
| | % recovery | | | | 40 | 32 | 63 | 32 | <0.1 |
| | Mg N/ml | 0.447 | | | | 0.021 | 0.027 | 0.035 | 0.042 |
| 3 | $LD_{50}$ (neg. log) | 6.3 | | | 5.8 | 5.8 | 5.9 | 6.0 | |
| | % recovery | | | | 32 | 32 | 40 | 50 | |
| | Mg N/ml | 0.504 | | | 0.034 | 0.050 | 0.055 | 0.049 | |

TABLE II.
Comparison of Virus Recoveries by Alcohol Precipitation at Different Temperatures.

| | | Methanol | | | Ethanol | | |
|---|---|---|---|---|---|---|---|
| Temp. of alcohol | % conc. of precipitant | $LD_{50}$ (neg. log) | % recovery | Mg N/ml | $LD_{50}$ (neg. log) | % recovery | Mg N/ml |
| –40°C | 0 (control) | 6.4 | *100 | .588 | 6.4 | 100 | .588 |
| | 10 | 6.0 | 40 | .012 | 6.0 | 40 | .024 |
| | 25 | 6.5 | 100 | .054 | <2.0 | <.1 | .066 |
| | 40 | <2.0 | <.1 | .090 | <2.0 | <.1 | .064 |
| 0°C | 0 (control) | 6.4 | 100 | .588 | 6.4 | 100 | .588 |
| | 10 | 5.5 | 13 | .050 | 5.6 | 16 | .021 |
| | 25 | 6.0 | 40 | .045 | <2.0 | <.1 | .064 |
| | 40 | <2.0 | <.1 | .070 | <2.0 | <.1 | .051 |

* The control $LD_{50}$ is given a value of 100%. The per cent recoveries of precipitated suspensions are all based on this figure. The control for both the ethanol and methanol series is the same virus suspension.

0.5 log, indicating recoveries between 63 and 32%, respectively, of the original virus material. Methanol concentrations of 40% total volume were in all cases definitely destructive to the virus.

*Precipitation of allantoic fluid with absolute ethanol as compared to methanol (at –40°C and 0°C).* In view of the poor recovery values obtained with methanol (–40°C), it was decided to make comparative tests using absolute ethanol as the precipitant at both –40°C and 0°C. To measured aliquots of the virus chilled to 0°C were added varying concentrations of the alcohol previously chilled to either (1) below –40°C in an acetone-dry ice bath, or (2) 0°C in an ice-water bath. The results are shown in Table II.

It can be seen that when the precipitations were performed at either temperature, a concentration of 25% ethanol was toxic, whereas fair or good recoveries were obtainable with methanol at the same concentration. At the 40% level, both alcohols resulted in a marked inactivation of the virus at either temperature. The probable greater denaturation of virus protein resulting from ethanol activity is in agreement with similar observations made by Liu *et al.*[7] on denaturation of serum proteins. The results of total nitrogen determinations indicated that similar purifications resulted whether ethanol or methanol was used.

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[7] Liu, Szu-Chih, and Wu, Hsien, *Chinese J. Physiol.*, 1937, **11**, 315.

In view of the observations made by Lazarus[8] on the comparative susceptibility of psittacosis virus to trypsin, attempts were made to digest the proteins of infected allantoic fluid by preliminary treatment with this enzyme. Although the virus withstood the action of the trypsin, it was not possible to demonstrate any increased purification when the digestion was done prior to alcohol precipitation.

In the course of our studies on the mechanism of the precipitation it became increasingly evident that, at least in the case of psittacosis virus, the sedimentation of the virus during the alcohol procedure was in all probability due to centrifugation (3500 rpm/30 minutes) and not to a virus protein precipitation. It was apparent also that preliminary centrifugation of infected allantoic fluids at 1500 rpm for 15 minutes was sedimenting not only large particulate matter, but a considerable proportion of the virus as well. This was especially true when the harvested allantoic fluid contained large quantities of insoluble material. Aliquots of original infected fluid, not subjected to any preliminary centrifugation, were precipitated with absolute methanol at –76°C and centrifuged at varying speeds from 1500 to 3500 rpm for 30 minutes, after which the sediments were suspended in phosphate buffer and tested for virus activity as previously described. A second virus preparation, previously purified by centrifugation,† was also subjected to the same alcohol procedure. Aliquots of the same

TABLE III.

Comparison of Virus Recoveries by Centrifugation and by Alcohol Precipitation.

| Virus | Speed of centrifugation (rpm) | Alcohol precipitation* $LD_{50}$ (neg. log) | Alcohol precipitation* % recovery | Alcohol precipitation* Mg N/ml | Centrifugation control $LD_{50}$ (neg. log) | Centrifugation control % recovery | Centrifugation control Mg N/ml | Uncentrifuged refrigerated control $LD_{50}$ (neg. log) | Uncentrifuged refrigerated control Mg N/ml |
|---|---|---|---|---|---|---|---|---|---|
| Original allantoic fluid | 1500 | 7.1 | 100 | .053 | 7.0 | 100 | .050 | | |
| | 2500 | 7.0 | 100 | .052 | 7.5 | 100 | .034 | | |
| | 3500 | 7.1 | 100 | .052 | 7.4 | 100 | .052 | 7.0 | .548 |
| Partially purified allantoic fluid | 1500 | 6.1 | 13 | .017 | 6.1 | 14 | .012 | | |
| | 2500 | 6.6 | 40 | .011 | 6.7 | 50 | .011 | | |
| | 3500 | 6.9 | 80 | .012 | 6.8 | 63 | .013 | 7.0 | .016 |

* All aliquots were precipitated with 25% by volume of absolute methanol.

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[8] Lazarus, A. S., and Meyer, K. F., *J. Bact.*, 1939, **38**, 121.

† Preliminary purification was accomplished as follows: original infected allantoic fluid was spun at 4000 r.p.m. for 60 minutes in a refrigerated angle-head centrifuge. The supernatant fluid was discarded and the sediment resuspended to approximately one-half the original volume in phosphate buffer, shaken with glass beads for several minutes, and centrifuged at 2000 r.p.m. for 15 minutes. The supernatant fluid was removed, spun again at 4000 r.p.m. for 60 minutes and the sediment resuspended to the original volume in buffer solution. This procedure removes most of the extraneous protein and cell debris without significant loss of active virus.

parent pools, not treated with alcohol, were included as controls.

Table III shows the results obtained in terms of the virus recovery and N content. It is apparent that when such preparations of original allantoic fluid are subjected to centrifugation alone, at speeds even as low as 1500 rpm, the virus is sedimented and recoverable in its entirety, within the limits of the error of the methods employed. Several virus samples indicated greater than 100% recovery, and it is believed that these are the results of disaggregation of virus clumps normally present in the original allantoic fluid. When a partially purified virus suspension was employed, the centrifugal force necessary to bring down virus activity was definitely increased but the addition of alcohol did not augment the recovery of virus. The N content of the partially purified virus suspension (Table III, under Refrigerated Control) is further shown to be lower than in the original allantoic fluid preparations treated with alcohol. The original fluid, which was subjected only to varying speeds of centrifugation from 1500 to 3500 rpm for 30 minutes, demonstrates as great a reduction in non-viral N as those preparations undergoing alcohol precipitation. Therefore, it must be concluded that the alcohol did not act as a virus precipitant with this agent and that the primary factor in sedimentation and purification by this procedure was the simple centrifugation employed in the process.

*Summary*. Experiments are reported on the effect of methanol and ethanol on the viability and purification of psittacosis virus. A comparison of this technic with differential centrifugation is presented.

The results suggest that the separation of virus from crude allantoic fluid was dependent on a non-specific adsorption of the virus on particulate matter either present originally in the allantoic fluid or brought out of solution by the alcohol. With virus suspensions first purified by centrifugation, the speed of centrifugation and not the precipitation by alcohol was the determining factor in the recovery of virus.

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### 16670

## Changes in Ascorbic Acid Metabolism of the Rat During Infection with *Trypanosoma hippicum*.*

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Investigation of the biochemical changes occurring in the host during trypanosomiasis has been directed primarily toward the observation of changes in carbohydrate metabolism.[1] There have been some studies, however, which indicate that the ascorbic acid metabolism of the host is affected during infection. Scheff and Csillag[2] showed that the ascorbic acid content of the liver and blood of guinea pigs is lowered during infection with *Trypanosoma brucei*. Ascorbic acid administration was reported by Perla[3] to increase the resistance of guinea pigs to infection with *T. brucei*, but not to alter the course of the infection in mice. These findings supported the report made by Borghi[4] that scorbutic guinea pigs showed an increased susceptibility to infection with *T. brucei.*

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* The work presented in this paper was supported by a grant from the U. S. Public Health Service.

In partial fulfillment of the requirements for the degree of Master of Science, Department of Pharmacology, University of Chicago.

[1] VonBrand, T., *Quart. Rev. Biol.*, 1938, **13**, 41.

[2] Scheff, G., and Csillag, Z., *Arch. f. exp. Path. Pharmakol.*, 1938, **188**, 467.

[3] Perla, D., *Am. J. Hyg.*, 1937, **26**, 374.

The present study was undertaken to investigate alterations in the content of the tissue ascorbic acid of rats infected with *T. hippicum.* The distribution of oxidized and reduced ascorbic acid was studied in the hope that analysis for both forms would yield some information leading to a better understanding of the biochemical changes observed during trypanosomiasis.

*Methods and Materials.* Male Sprague-Dawley rats weighing 200-300 g were kept on a uniform diet[5] 3 weeks previous to use in order to attain a constant level of ascorbic acid in the tissues. The animals were divided into two groups. The first, or control, group was sacrificed at the end of the 3 weeks feeding period, while the second group was infected after this feeding period and then maintained on the same diet during the course of infection. Both normal and infected animals were fasted 24 hours before their tissues were taken for analysis. The strain of *T. hippicum* used[†] was maintained by passage through rats, which were infected by intraperitoneal injection of the trypanosomes suspended in Ringer-Locke solution at pH 7.4. About 36 hours after infection the level of the parasitemia was determined by hemocytometer. From this information is was possible to predict the time of death to within 6 hours of its occurrence which was, on the average, 60 hours after infection. The infected rats were sacrificed after pre-mortal symptoms appeared, but before the terminal convulsions occurred.

Samples of liver, spleen, adrenal, skeletal muscle and plasma were taken for analysis. In the assay of the adrenals, one gland was used for the determination of oxidized ascorbic acid and the other for total ascorbic acid. Plasma, free of trypanosomes, was obtained by differential centrifugation of heparinized blood collected after decapitation of the animals. The tissues were homogenized[6] in a 5% trichloroacetic acid for total ascorbic acid, or in 6% metaphosphoric acid containing 0.5% thiourea for dehydroascorbic acid analyses. Samples of plasma were added to similar acid solutions. Dehydroascorbic acid was measured in the acid extracts by the method of Roe and Oesterling[7] modified by Herrmann and DuBois,[8] and total ascorbic acid was measured by the method of Bessey, Lowry, and Brock.[9] The spectrophotometric measurements were carried out on a Coleman spectrophotometer at 520 mμ wave length. Values for reduced ascorbic acid were calculated by subtracting the values for dehydroascorbic acid from those of the total.

All values for tissue ascorbic acid were expressed as mg of ascorbic acid per g of dry tissue. There was no significant difference between the water content of the tissues of the infected rats and that of the control rats. The results of the plasma analyses were expressed as mg of ascorbic acid per g of plasma. Means were calculated for both the control and infected animals, and the significance of the results was evaluated by calculating the "t" values between the means of the experimental and control data, in which $t = \frac{m_1^2 - m_2^2}{\sqrt{\epsilon_1^2 + \epsilon_2^2}}$.[10]

*Results.* The figures in part 1 of the table show that a large decrease was observed in the total ascorbic acid content of the spleen. The greatest proportionate decrease took place in the reduced fraction, while the dehydroascorbic acid showed a slight, but not very significant, decrease in the infected animals. In the course of these experiments it was observed that the ratio of the wet weight of the spleen to the total body weight increased from 1.94 x $10^{-3}$ in the controls to 5.46 x $10^{-3}$ in the infected rats, an increase of 281% in the spleen to body weight ratio. When the total amount of ascorbic acid was calculated from the weight of the total spleen and the concentration of ascorbic acid in mg per g of *wet* tissue, if was found to increase only 175%. This indicated that the decrease observed in the spleen was an actual diminution in the ascorbic acid content, and was not due to a dilution of the ascorbic acid normally present as the result of splenic hypertrophy.

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[4] Borghi, B., *Atti accad. Lincei,* 1933, **17**, 665.

[5] Hasch, Z., and Hajdu, I., *Pflügers Arch.,* 1939, **241**, 507.

[†] The strain of *T. hippicum* used in these experiments was kindly supplied by Dr. M. H. Soule, University of Michigan.

[6] Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.,* 1936, **114**, 495.

[7] Roe, J. H., and Oesterling, M. J., *J. Biol. Chem.,* 1944, **152**, 511.

[8] Herrmann, R., and DuBois, K., unpublished.

[9] Bessey, O., Lowry, O., and Brock, M. J., *J. Biol. Chem.,* 1947, **168**, 197.

[10] Burn, J. H., Biological Standardization, Oxford University Press, London, 1937, 29.

The results of the ascorbic acid analyses on the adrenals are presented in part II of the table. The total ascorbic acid of the adrenals of the infected animals decreased significantly. The primary alteration occurred in the reduced fraction, while the dehydroascorbic acid showed no change from normal. Since the ascorbic acid of the adrenal is concentrated in the cortex, the change represented by these results would be more pronounced if the determination were based on the cortex alone, rather than upon the entire adrenal.

The changes which took place in the liver ascorbic acid are shown in part III of the table. The total ascorbic acid decreased during infection. As in the spleen and adrenal, the greatest proportionate decrease occurred in the reduced form. The dehydroascorbic acid of the liver of the infected animals was also less than that observed in the controls, but this decrease was not statistically significant.

The ascorbic acid concentration of the muscle was not greatly affected by infection with *T. hippicum*. It can be seen from part IV of the table that, although the muscle dehydroascorbic acid increased slightly, the significance of the increase ($t = 2.9$) is open to question.

The figures in part V of the table show that the ascorbic acid of the plasma was elevated above normal. Unlike the tissues discussed previously, both fractions were increased to the same extent. The dehydroascorbic and reduced ascorbic acid each increased 100% in the plasma of the infected rats.

*Discussion.* The lowered concentration of reduced ascorbic acid in the spleen and adrenal, without an equivalent decrease in oxidized ascorbic acid, indicates that there was an interference in the oxidation-reduction processes of the host during infection, which may have been responsible for the changes observed in the distribution of ascorbic acid in the infected animals. These findings are comparable to those of Scheff and Csillag[2] who found that the reduced glutathione decreased while the total glutathione did not change in the blood and liver of guinea pigs infected with *T. brucei*. To a lesser extent, a similar decrease took place in the ratio of reduced to oxidized ascorbic acid in the muscle, because of the increase observed in dehydroascorbic acid. In the liver and plasma, the normal ratio was not measurably altered.

The decrease in ascorbic acid in all tissues studied, except muscle, accompanied by an increase in plasma ascorbic acid, suggested that the metabolite may have diffused from the tissues to the blood. This explanation does not seem wholly adequate, since a greater decrease took place in the reduced fraction in the depleted tissues, while both forms of ascorbic acid increased in the plasma.

Our knowledge of the metabolic requirements of trypanosomes is limited, but it is of interest to note that Groat and Mora[11] found that their strain of *T. cruzi* required reduced ascorbic acid for cultivation *in vitro*. Further investigation of the changes produced in the oxidation-reduction systems of the host by *T. hippicum* may reveal the significance of the alterations in the distribution of ascorbic acid observed and their relation to the symptomology of the infection.

*Summary.* Dehydroascorbic and total ascorbic acid were measured in the liver, spleen, adrenals, muscle, and plasma of rats infected with *T. hippicum* and the reduced ascorbic acid content of these tissues was calculated.

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[11] Groat, H., and Mora, C., *Anales soc. biol. Bogóta*, 1947, **2**, 188.

TABLE I. The Effect of *T. hippicum* Infection on Ascorbic Acid Content of Rat Tissues. Ascorbic acid content is expressed as mg per g of dry tissue. In the spleen, adrenal, liver and muscle, 7 control animals and 7 infected animals were used. In the analyses made upon the plasma, 2 separate groups of 8 animals each were used.

| | Animal No. | Total mg/g | | Dehydro. mg/g | | Reduced mg/g (Total-dehydro.) | |
|---|---|---|---|---|---|---|---|
| | | Control | Infected | Control | Infected | Control | Infected |
| Spleen I | 1 | 3.22 | 1.75 | 0.59 | 0.34 | 2.63 | 1.41 |
| | 2 | 3.04 | 2.14 | 0.46 | 0.27 | 2.58 | 1.87 |
| | 3 | 2.83 | 1.64 | 0.20 | 0.34 | 2.63 | 1.30 |
| | 4 | 3.14 | 1.72 | 0.35 | 0.19 | 2.79 | 1.53 |
| | 5 | 3.00 | 1.72 | 0.30 | 0.23 | 2.70 | 1.49 |
| | 6 | 2.79 | 1.96 | 0.36 | 0.26 | 2.43 | 1.70 |
| | 7 | 2.92 | 2.07 | 0.33 | 0.26 | 2.59 | 1.81 |
| | mean | 2.99 | 1.86 | 0.37 | 0.27 | 2.62 | 1.59 |
| | $\epsilon$ | 0.06 | 0.07 | 0.05 | 0.02 | 0.06 | 0.08 |
| | t* | 11.9 | | 2.0 | | 10.3 | |
| Adrenal II | 1 | 12.75 | 11.41 | 0.99 | 1.03 | 11.76 | 10.38 |
| | 2 | 14.96 | 8.77 | 1.24 | 0.52 | 13.72 | 8.25 |
| | 3 | — | 11.65 | 1.26 | 1.39 | — | 10.25 |
| | 4 | 12.67 | 9.02 | 0.83 | 0.98 | 11.84 | 8.04 |
| | 5 | 13.50 | 8.31 | 1.02 | 0.82 | 12.48 | 7.49 |
| | 6 | 10.98 | 10.25 | 0.91 | 1.17 | 10.07 | 9.08 |
| | 7 | 13.74 | 8.77 | 0.71 | 0.74 | 13.03 | 8.03 |
| | mean | 13.10 | 9.70 | 0.99 | 0.95 | 12.15 | 8.79 |
| | $\epsilon$ | 0.54 | 0.51 | 0.08 | 0.11 | 0.52 | 0.43 |
| | t | 4.6 | | 0.31 | | 5.0 | |
| Liver III | 1 | 1.22 | 1.01 | 0.29 | 0.32 | 0.93 | 0.79 |
| | 2 | 1.38 | 0.66 | 0.23 | 0.09 | 1.12 | 0.57 |
| | 3 | 1.28 | 1.03 | 0.35 | 0.23 | 0.93 | 0.80 |
| | 4 | 1.10 | 0.84 | 0.12 | 0.08 | 0.98 | 0.76 |
| | 5 | 1.59 | 0.80 | 0.15 | 0.08 | 1.43 | 0.72 |
| | 6 | 1.08 | 0.78 | 0.17 | 0.11 | 0.91 | 0.67 |
| | 7 | 1.17 | 0.67 | 0.13 | 0.13 | 1.04 | 0.54 |
| | mean | 1.26 | 0.83 | 0.21 | 0.15 | 1.05 | 0.69 |
| | $\epsilon$ | 0.07 | 0.06 | 0.03 | 0.03 | 0.07 | 0.04 |
| | t | 4.90 | | 1.4 | | 4.5 | |
| Muscle IV | 1 | 0.18 | 0.23 | 0.06 | 0.16 | 0.12 | 0.07 |
| | 2 | 0.17 | 0.33 | 0.08 | 0.10 | 0.09 | 0.23 |
| | 3 | 0.20 | 0.20 | 0.07 | 0.14 | 0.12 | 0.07 |
| | 4 | 0.25 | 0.20 | 0.07 | 0.06 | 0.18 | 0.14 |
| | 5 | 0.20 | 0.24 | 0.07 | 0.06 | 0.12 | 0.17 |
| | 6 | 0.20 | 0.20 | 0.07 | 0.09 | 0.12 | 0.11 |
| | 7 | 0.18 | 0.20 | 0.07 | 0.09 | 0.11 | 0.11 |
| | mean | 0.20 | 0.23 | 0.07 | 0.10 | 0.12 | 0.13 |
| | $\epsilon$ | 0.01 | 0.02 | 0.003 | 0.01 | 0.01 | 0.02 |
| | t | 1.8 | | 2.9 | | 0.44 | |
| Plasma V† | 1 | 0.015 | 0.041 | 0.010 | 0.017 | 0.005 | 0.024 |
| | 2 | 0.025 | 0.042 | 0.016 | 0.032 | 0.009 | 0.010 |
| | 3 | 0.017 | 0.050 | 0 012 | — | 0.005 | — |
| | 4 | 0.023 | 0.046 | 0.009 | 0.028 | 0.014 | 0.018 |
| | 5 | 0.019 | 0.042 | 0.014 | 0.033 | 0.005 | 0.009 |
| | 6 | 0.026 | 0.050 | 0.015 | 0.039 | 0.011 | 0.011 |
| | 7 | 0.016 | 0.051 | 0.008 | 0.021 | 0.008 | 0.030 |
| | 8 | 0.020 | 0.032 | 0.017 | 0.020 | 0.003 | 0.012 |
| | mean | 0.020 | 0.044 | 0.013 | 0.027 | 0.008 | 0.016 |
| | $\epsilon$ | 0.002 | 0.003 | 0.001 | 0.003 | 0.001 | 0.003 |
| | t | 9.6 | | 4.4 | | 2.7 | |

$$^{*}t = \frac{m_1 - m_2}{\sqrt{\epsilon_1^2 + \epsilon_2^2}}$$

† mg ascorbic acid/g of plasma.

The liver, spleen and adrenals showed significant decreases in reduced ascorbic acid content. The dehydroascorbic acid content of the muscle was increased slightly and both dehydroascorbic and reduced ascorbic acid of the plasma were increased two-fold.

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### 16671

## Effect of Environmental Temperature Upon Clotting Time of Whole Blood.

MURRAY WEINER AND SHEPARD SHAPIRO

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The *in vitro* clotting time of normal whole blood, as reported by various workers, has a wide range of activity, varying from one minute to over 15 minutes.[1] There are several factors responsible for the wide variations. One important consideration is the method of obtaining blood samples. When finger puncture methods are used, there is a variable degree of contamination with thromboplastic tissue substances. With venous blood, the tissue substances are important only when venipuncture is not smooth. Another major variable is the degree of trauma and surface contact to which the blood is subjected during the determination. When the venipuncture is clean, and the apparatus and manipulations are standardized so that traumatic factors are kept constant, the only remaining significant extrinsic variable is the temperature. It is the purpose of this paper to indicate the magnitude of the effect of room temperature on the clotting time of whole blood.

In order to standardize the trauma factor and retain a precise endpoint, a rotating tube method for clotting time has been developed.[1] By this method 0.5 ml of venous blood is promptly placed in the bulbous end of a tube rotated around its long axis at a slow constant rate (one r.p.m.) by an electric motor. When the blood clots, the fibrous strands adhere to the ascending wall of the tube and are thus readily detected. The advantages of this method are that the degree of trauma and surface contact is constant and mechanically determined, and the endpoint is precise.

In order to determine the influence of temperature on the clotting time by this method, three identical sets of apparatus were set up at three different temperatures. One-half ml aliquots of the same samples of blood were delivered into bulbous tubes and the clotting time (fibrin appearance time) determined at the 3 different temperatures. The

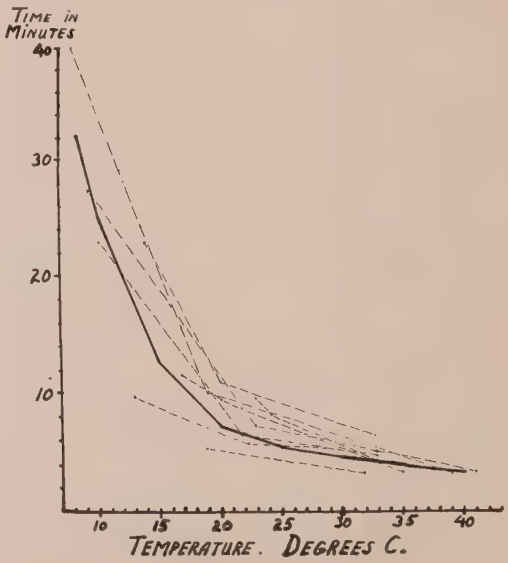

FIG. 1.
Relationship of clotting time to room temperature.

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[1] Weiner, M., and Shapiro, S., *J. Lab. and Clin. Med.*, 1947, **32**, 1037.

blood samples were taken from patients chosen at random. In Fig. 1 the temperatures at which each clotting time was determined is plotted against the corresponding clotting time, and points determined with the same sample of blood are connected by broken lines. The solid line represents the estimated average slope of these broken lines. Its position in the figure is based on the average normal value of 5 min. 28 sec. previously determined at about 26°C. At this temperature, 4 to 6 3/4 min. is considered the normal range. It is obvious from the curve that winter-summer variations of room temperature, which may range from 19°C to 37°C in temperate climates, can result in considerable changes in the normal range of clotting time.

It is recommended that a curve such as shown in Fig. 1 be used to evaluate clotting time results whenever this determination is performed at room temperature.

*Summary.* The effect of room temperature on the clotting time of whole blood determined by the rotating tube method is described. The clotting time may be significantly prolonged in cool environments, and shortened in warm weather. A curve is described to correct for this factor.

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### 16672

## Pteroylglutamic Acid Deficiency in Mice: Hematologic and Histologic Findings.*

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The production of a deficiency of pteroylglutamic acid (PGA) can be accomplished in various mammalian species by feeding a purified diet containing the known essential vitamins, excluding PGA, to which has been added either succinylsulfathiazole, a PGA antagonist, or both. In the deficient state the animals have shown varying degrees of reduction of all circulating elements in the blood stream. Reports of bone marrow findings have been conflicting and incomplete and have not been reported previously for mice.

*Procedure.* Deficiency may be established in weanling mice more quickly than in adults. Weanling animals of a Swiss strain with an average weight of about 20 g were placed in wire mesh cages and were allowed distilled water ad libitum. They were fed unlimited quantities of a PGA free diet of the following percentage composition:

| | |
|---|---|
| Glucose (''Cerelose'') | 59.1 |
| Casein (''Labco'' vitamin free) | 20.0 |
| Cellulose (''Cellu-flour'' or ''Ruffex'') | 5.0 |
| Salts USP (Smaco) | 3.8 |
| Accessory salts* | 0.2 |
| Choline chloride | 0.2 |
| Inositol | 0.1 |
| Thiamine hydrochloride | 0.0005 |
| Riboflavin | 0.001 |
| Pyridoxine hydrochloride | 0.0005 |
| Niacinamide | 0.005 |
| Calcium pantothenate | 0.0055 |
| Ascorbic acid | 0.01 |
| 2-methyl-1, 4-napthoquinine | 0.001 |
| Biotin | 0.00001 |
| Hydrogenated vegetable oils (''Primex'') | 8.0 |
| Corn oil containing vit. A, D, and E† | 2.0 |

* Accessory salts contained in 0.2 g (expressed in mg): KCl, 100; NaCl, 87.2; $FeSO_4$, 10; $MnSO_4$, 1; $ZnSO_4 \cdot 7H_2O$, 1; $CuSO_4 \cdot 5H_2O$, 1; NaI, 0.3; NaF, 0.1; $CoCl_2 \cdot 6H_2O$, 0.01.

† Two grams of the corn oil preparation contained vitamin A, about 4500 units; vitamin D, about 560 units; and mixed tocopherols, about 2.7 mg.

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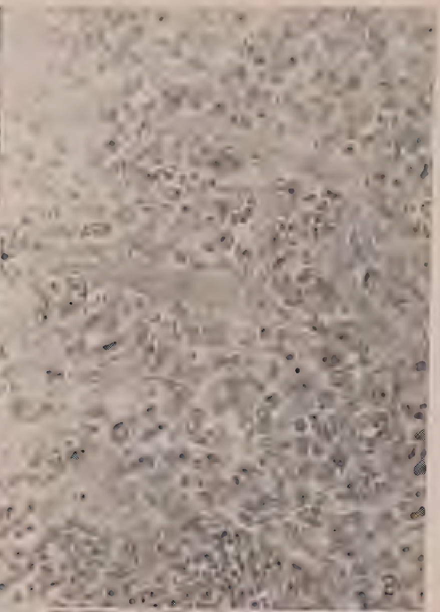
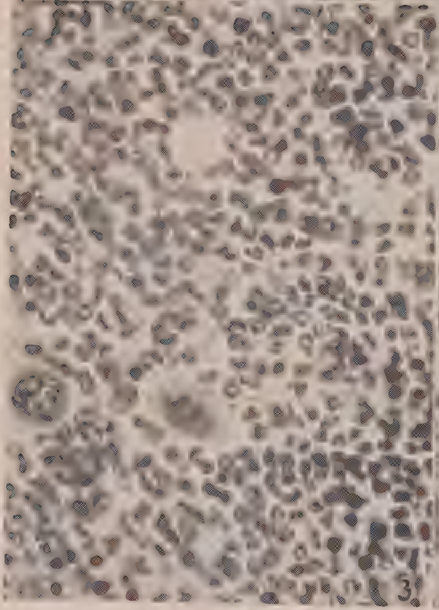
* This research was made possible by a grant from the Price McKinney Memorial Fund and is part of a study aided by a grant from the United States Public Health Service.





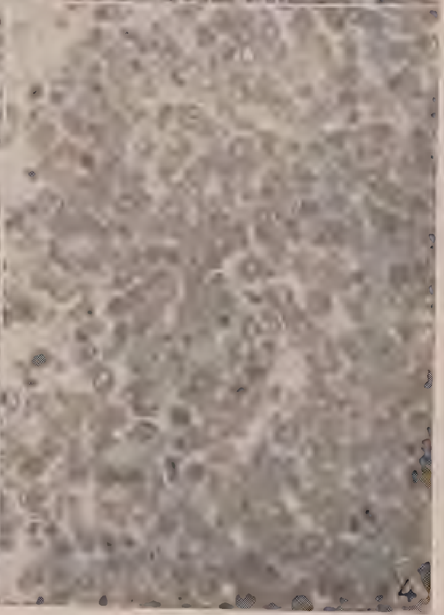


PLATE 1. Normal Spleen. ×246. Note the degree of cellularity and the numerous nucleated red blood cells and megakaryocytes. Actually this spleen is from a mouse that had been deficient and was cured by the administration of PGA.

PLATE 2. Deficient Spleen. ×246. The cellularity is much reduced. Nucleated red blood cells and megakaryocytes have practically disappeared. Extensive deposits of hemosiderin are present but these cannot be appreciated in the absence of color-reproduction. The coarsely granular appearance of some of the cells is due to hemosiderin.

PLATE 3. Normal Marrow. ×410. Note the presence of numerous nucleated red blood cells, polymorphonuclear leucocytes, and megakaryocytes. The absence of fat is normal in the mouse.

PLATE 4. Deficient Marrow. ×410. Large immature cells with nucleoli and mitoses predominate. Almost no polymorphonuclear leucocytes or nucleated red cells are seen and megakaryocytes have disappeared.

Five-tenths percent of a crude antagonist of PGA (Lederle N-67A)[1†] was added to the diet. In a preliminary experiment succinylsulfathiazole[‡] also was added but it was found that the majority of animals died before the desired evidences of deficiency had developed. Therefore the sulfonamide was withheld for the first month and then added at a level of 1.0% in the diet. With this modification only a very small percentage of the animals died in the preliminary stages of the experiment. On this regimen, deficiency was established in all animals within 50 to 60 days. A number then were killed for histologic studies. The remainder of the group was divided into 3 sub-groups and given varying amounts of PGA in the form of solutions of 'Folvite' (Lederle). Daily subcutaneous injections of 20, 2, and 0.2 $\mu$g were used. Care was taken to protect the solutions from exposure to sources of ultra-violet radiation. At weekly intervals the animals were weighed and white blood cell counts were done. Normally leucocyte counts of mice are not less than 6000 per cmm. When levels below 4000 were reached, it was considered that PGA deficiency was established. Red blood cell counts were then done; and, during the period of treatment with PGA, daily reticulocyte counts also were performed.

*Hematologic Findings.* After 30 to 40 days of the above regimen the white blood cell counts begin to fall to low normal levels. By 50 days many counts are below the minimal normal level of 6000, and by 60 days practically all the counts are below 4000 with some as low as 1000. Both lymphocytes and granulocytes participate in this leucopenia. There is no consistent change in the differential count; the normal lymphocyte-to-granulocyte ratio is preserved. Red blood cell counts done at the time when the leucocytes had fallen to levels of less than 6000 per cc showed that a moderate to marked anemia had developed. From a normal count of about 10 million erythrocytes per cc the count was usually reduced to 4 to 6 million with some counts as low as 2 million per cc.

The entire blood picture may be restored rapidly to normal by the parenteral administration of PGA while the animals are still on the dietary regimen with the added antagonist and succinylsulfathiazole. A maximal response was obtained by giving daily subcutaneous injections of 20 $\mu$g of a solution of 'Folvite' (Lederle). There is a reticulocyte rise in 2 days which usually reaches a maximum of about 30% in 7 days. The erythrocyte counts become normal within 15 to 20 days. Also there is a very rapid regeneration of leucocytes, normal counts being obtained within 3 to 5 days. More remarkable is the fact that abnormally high white counts may occur. In one animal the initial leucocyte count of 2000 per cc rose to 57,000 per cc 3 days after PGA was started, and to 85,000 per cc 3 days later; after another 3 days it was below the normal maximum of 25,000 per cc.

During the first month prior to the administration of succinylsulfathiazole there is a moderate gain in weight. On the addition of this drug to the purified diet the mice soon cease to gain in weight and many animals show losses of a few grams. When PGA is given, weight gain is resumed at a rapid rate.

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[1] Franklin, A. L., Stokstad, E. L. R., Belt, M., and Jukes, T. H., *J. Biol. Chem.*, 1947, **169**, 427.

[†] Generously supplied by the Lederle Laboratories Division of the American Cyanamid Co.

[‡] Kindly furnished by the Medical Research Division of Sharp and Dohme, Inc.

Also the ruffled fur characteristic of the deficient state disappears.

*Histologic Findings.* See Plates 1-4. The spleen of normal mice is an active hematopoietic organ. Many megakaryocytes are present and numerous foci of nucleated red blood cells are scattered diffusely through the pulp. There are a few polymorphonuclear leucocytes and occasionally immature cells. Mitoses are rarely seen. In PGA deficiency the hematopoietic activity of the spleen practically ceases. Megakaryocytes and nucleated red cells may entirely disappear. Polymorphonuclear leucocytes are decreased in number. The pulp becomes sparsely cellular and shows large amounts of hemosiderin, both in the free form and phagocytized by macrophages. As in pernicious anemia, the hemosiderin probably represents iron released by the normal breakdown of erythrocytes. In PGA deficiency it is not utilized because of the decreased formation of erythrocytes. The splenic follicles did not show any consistent change from the normal. Treatment with PGA restores the histology of the spleen to normal, although some of the hemosiderin may persist. In a few animals, in the deficient state, there was a moderate increase in the number of immature cells in the splenic pulp in addition to the findings described above. This was never as prominent in the spleen as in the bone marrow.

The marrow of normal mice contains practically no fat. The cellular elements consist of nucleated red blood cells, megakaryocytes, polymorphonuclear leucocytes, a few immature cells, a few lymphocytes, and rare mitoses. In PGA deficiency a very marked change occurs. The marrow becomes solidly packed with large immature cells containing nucleoli and showing numerous mitoses. Only a few identifiable nucleated red cells remain. Polymorphonuclear leucocytes and megakaryocytes may disappear entirely. The picture is one of maturation arrest with overproduction of immature cells. Positive identification of these immature cells has not been made. They have not been identified as either myeloblasts or megaloblasts; it seems likely they fall into the category of primitive blastic cells. After treatment with PGA the marrow cannot be distinguished from that found in normal mice.

In a few of the animals small areas of necrosis were present in the liver. Both in this experiment and in preliminary experiments these areas of necrosis had been found in animals which had died before the development of the PGA deficiency as evidenced by leucopenia and anemia.

*Discussion.* There is general agreement that PGA deficiency produces a reduction of all circulating elements in the blood streams of various animals and that this reduction may be corrected by the administration of PGA. The appearance of immature elements in the peripheral blood has not been described. Reports on the histologic findings have been variable. Bethell, Swenseid, and Rosenman[2] using rats found that the nucleated cell count of the marrow fell slightly below normal. The majority of the marrow cells were undifferentiated primitive forms, with relative increase in myeloblasts and early erythroblasts. Myelocytes, late erythroblasts and megakaryocytes practically disappeared. The situation could be corrected by extracts of liver or yeast containing folic acid. Gross, Axelrod and Bosse[3] using rats, stated that the marrow findings varied from almost complete aplasia to intense hyperplasia and concluded that the pathologic changes included progressive hypoplasia of the bone marrow. Endicott, Daft and Ott,[4] using rats, described a decrease of all elements of the marrow, lymphoid exhaustion of the spleen, and absence of splenic hematopoiesis. They observed a pancytopenia of the marrow without maturation arrest or hyperplasia of immature elements. Doan,[5] working with monkeys, described hypoplasia of the marrow with resulting peripheral panhematopenia; detailed studies of the marrow were not included in the report. Heinle, Welch, George, Epstein, and Pritchard,[6] using swine, found megaloblastic hyperplasia of the marrow. Franklin, Stokstad, Belt, and Jukes,[1] using rats, observed an increase of nucleated red blood cells in the marrow, an increased number of blast cells, and a marked reduction of the polymorphonuclear leucocytes. They stated that the maturation of cells of the erythroid series and the production of mature granulocytes were seriously impaired. They did not stress hyperplasia with maturation arrest. Cartwright, Fay, Tatting, and Wintrobe,[7] using swine, found a reduction of polymorphonuclear leucocytes and metamyelocytes, a slight increase in earlier forms of the myeloid series, and a significant decrease in the leucocyte-erythrocyte ratio. They observed immature and nucleated red cells which they considered to be similar in many ways to those seen in the marrow of patients with pernicious anemia in relapse.

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[2] Bethell, F. H., Swenseid, M. E., and Rosenman, R. H., *J. Clin. Invest.*, 1944, **23**, 926.

[3] Gross, P., Axelrod, A. E., and Bosse, M. D., *Am. J. Med. Sci.*, 1944, **208**, 642.

[4] Endicott, K. M., Daft, F. S., and Ott, M., *Arch. Path.*, 1945, **40**, 364.

[5] Doan, C. A., *Am. J. Med. Sci.*, 1946, **212**, 257.

The exact role of PGA in the formation and maturation of blood cells has not been elucidated. Certainly it participates in an important way. If it were an essential growth factor necessary for the formation of the most primitive blood cells we would expect pancytopenia of bone marrow, rather than maturation arrest and hyperplasia of immature elements. It may be inferred, then, that PGA does not participate in the production of the most primitive blood elements, but rather that it must be present to allow maturation beyond the most primitive stages. In pernicious anemia in relapse, and in some cases of agranulocytosis, a similar although an apparently selective maturation arrest with hyperplasia of immature elements is seen. The production of primitive elements is not abolished and may even be in excess. It has been postulated that in red cell formation the role of the anti-pernicious anemia factor of liver is to act in conjunction with PGA or its conjugates to bring about the maturation of red blood cells beyond the megaloblastic stage. This would fit into our hypothesis that PGA may not be necessary for the formation of the most primitive blood elements, but that it is concerned with reactions essential to maturation such as loss of nuclear material.

*Summary.* 1. A deficiency of pteroylglutamic acid in mice produces a reduction of all cellular elements of the circulating blood.

2. In the deficient state, the bone marrow of mice shows maturation arrest with hyperplasia of immature elements and marked reduction of more adult forms.

3. It is suggested that pteroylglutamic acid may not be necessary for the formation of the most immature blood elements, but must be present to allow maturation after these primitive elements are formed.

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[6] Heinle, R. W., Welch, A. D., George, W. L., Epstein, M., and Pritchard, J. A., *Proc. Central Soc. Clin. Research*, 1947, **20**, 7.

[7] Cartwright, G. E., Fay, J., Tatting, B., and Wintrobe, M. W., *J. Lab. and Clin. Med.*, 1948, **33**, 397.

## 16673 P

# Stability of Serum Ac-Globulin.*

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Serum Ac-globulin is a clotting factor found in the globulin fraction of blood serum[1,2] which accelerates the change of prothrombin to thrombin. This factor is present in plasma in a much less active form, plasma Ac-globulin,[3-7] which serves as a precursor to serum Ac-globulin. The role of Ac-globulin in the total clotting reaction has been described as follows:[1,2] The clotting reaction is initiated by thromboplastin, which, in the presence of calcium ions, interacts with prothrombin to form thrombin. The thrombin in turn alters the plasma Ac-globulin so that it becomes serum Ac-globulin. The latter then intensifies the reaction between thromboplastin and prothrombin. Clotting is then accomplished by the action of thrombin on fibrinogen.

In the developmental work on Ac-globulin, oxalated bovine plasma was used as a potent stable source of plasma Ac-globulin[8] and bovine serum[1] a stable source of serum Ac-globulin. In working with the sera of other species involving man, dog, and guinea pig, we have found that while plasma Ac-globulin is relatively stable, serum Ac-globulin is inactivated and largely disappears within a few hours after the blood is drawn. Rabbit serum Ac-globulin is similar to bovine serum Ac-globulin in that it remains stable for longer periods of time.

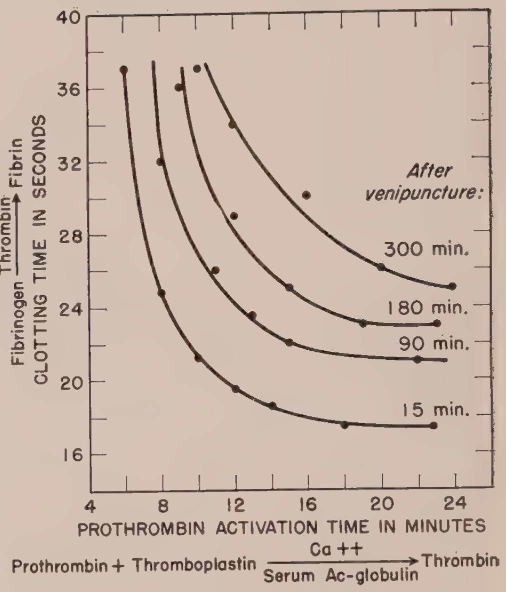

Fig. 1.
Change in Ac-globulin activity of human serum stored at 5°C.

In the study of the stability of serum Ac-globulin, blood was drawn and immediately centrifuged at 3,000 rpm for 10 minutes, and the serum was removed and stored at 5°C. Samples were analyzed for serum Ac-globulin by methods elsewhere described[1] after storage for periods of 15, 90, 180 and 300 minutes, as indicated in Fig. 1. In this procedure, human serum diluted 1,400 times was incubated for varying time intervals (as indicated on the horizontal axis, Fig. 1) with purified bovine prothrombin[9] and with optimal concentrations of thromboplastin and calcium.

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* Aided by a grant from the United States Public Health Service.

[1] Ware, A. G., and Seegers, W. H., *Am. J. Physiol.*, 1948, **152**, 567.

[2] Ware, A. G., Murphy, R. C., and Seegers, W. H., *Science*, 1947, **106**, 618.

[3] Fantl, P., and Nance, M., *Nature*, 1946, **158**, 708.

[4] Owren, P. A., *The coagulation of blood. Investigations on a new clotting factor*, Oslo, 1947.

[5] Owren, P. A., *Lancet*, 1947, **1**, 446.

[6] Ware, A. G., Guest, M. M., and Seegers, W. H., *J. Biol. Chem.*, 1947, **169**, 231.

[7] Ware, A. G., Guest, M. M., and Seegers, W. H., *Science*, 1947, **106**, 41.

[8] Murphy, R. C., Ware, A. G., and Seegers, W. H., *Am. J. Physiol.*, 1947, **151**, 338.

[9] Ware, A. G., and Seegers, W. H., *J. Biol. Chem.*, 1948, **174**, 565.

The amount of thrombin formed during this period was measured by the addition of fibrinogen and by observing the time required for a clot to form (vertical axis, Fig. 1). This observed time is an inverse measure of the amount of thrombin formed in the first reaction. The rate of thrombin production is proportional to the concentration of the Ac-globulin in the reacting medium. Thus, it may be seen that there is a definite decrease in serum Ac-globulin activity from the time that the blood was drawn. Within 300 minutes after blood was drawn less than one-third of the Ac-globulin activity remained. On standing at room temperature the loss of serum Ac-globulin activity was considerably more pronounced (not shown in Fig. 1).

One would naturally like to offer an explanation for these observed variations, but sufficient information is not available at this time. It has been shown that relatively large amounts of thrombin (30 units per cc or more) partially destroy the Ac-globulin activity of oxalated bovine plasma.[1] It is therefore possible that the instability of serum Ac-globulin in certain species may be due to the action of thrombin. Since the amount of thrombin present during and immediately following the clotting reaction is determined by the rate of thrombin formation and by the effective concentration of antithrombin, variations of these factors from one species to another may account for the differences in the stability of Ac-globulin activity. The stability of serum Ac-globulin may also be an inherent characteristic of the substance for each species.

The similarity in the stability of human serum Ac-globulin and that of Factor VI described by Owren[4] tends to confirm the suggestion that these two substances are identical.

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### 16674

## Effects of Diethylcholine and Ethionine on Growth Rate, Liver Fat, and Kidney Degeneration of Rats.

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It is now well established that a dietary deficiency of choline and methionine leads to an accumulation of fat in the liver, to the development of hemorrhagic degeneration of the kidney, and to a failure of young animals to grow on diets containing homocystine but lacking in labile methyl groups. The lipotropic action and kidney protection have been ascribed to the action of intact choline or choline-like molecules or their precursors, whereas growth on a low methionine diet containing homocystine is dependent upon the availability of labile methyl groups.

Diethylmethylhydroxyethyl ammonium chloride (diethyl choline)[1] and ethionine[2] have been reported to be toxic to rats on low methionine diets. These compounds are ethyl homologs of choline and methionine respectively, and their toxicity raised interesting questions as to the mechanisms by which they may interfere either with fat metabolism or with transmethylation. The present experiments were devised to investigate in the rat the influence of these two compounds on the three criteria of inadequate choline or methyl group intake-growth on a diet containing

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* The data are taken from a thesis presented by Virginia L. Hardwick to the Graduate School of the University of Southern California in partial fulfillment of the requirements for the degree of Master of Science.

[1] Moyer, A. W., and du Vigneaud, V., *J. Biol. Chem.*, 1942, **143**, 373.

[2] Dyer, H. M., *J. Biol. Chem.*, 1938, **124**, 519.

TABLE I.
Constitution of the Basic Diet.

| Constituent | % |
|---|---|
| Arachin | 18 |
| Cerelose | 65.2 |
| Osborne and Mendel salts | 4 |
| Crisco and Lard* | 10 |
| Vitamin mixture† | 2 |
| Supplement | 0.8 |

* The Crisco and lard was fortified with 6,000,000 U.S.P. units of vitamin A, 85,000 units of vitamin D, and 0.5 mg of alpha tocopherol per g of fat.

† The vitamin mixture had the following composition: cerelose, 95 g; riboflavin, 50 mg; thiamin hydrochloride, 25 mg; pyridoxine, 25 mg; nicotinic acid, 100 mg; calcium pantothenate, 50 mg; and inositol, 5 mg.

homocystine, deposition of liver fat, and hemorrhagic degeneration of the kidneys.

*Methods.* Diethyl choline was prepared by treating diethylaminoethanol in ether with one equivalent of methyl chloride at –50°C and allowing the condensation to proceed at room temperature at high pressure. Homocystine was prepared by the method of Patterson and du Vigneaud.[3] Ethionine was prepared from S-benzylhomocysteine according to the method of Dyer.[2]

The basic diet used had the composition shown in Table I. Arachin was selected for the protein of the basic diet since it has been shown to contain limited amounts of methionine. It was prepared from peanut meal by the method of Johns and Jones[4] and was found to contain 0.5% methionine. All diets were supplemented with 0.8% homocystine, ethionine, or methionine as indicated in the experimental protocols. Choline or diethylcholine were administered daily by mouth with a 0.25 ml syringe and a rounded needle. The animals objected to the administration of the supplements, but with some practice this could be achieved without loss. Rats of the University of Southern California strain weighing between 50 and 70 g were employed throughout. The experiments were continued for 12 days with the food consumption and weight changes being determined every second day.

Liver lipids were determined by extracting the dried samples with diethyl ether in continuous extractors, the total lipids being determined gravimetrically. The choline in the liver lipids were determined by the Reinecke salt method of Engle[5] after hydrolysis with barium hydroxide. This method does not distinguish between choline and its monoethyl, diethyl or triethyl analogues. The kidneys were decapsulated and fixed in Zenkers solution, and sections embedded, sectioned and stained with iron hematoxylin.

*Results.* The results of the experiments are given in Table I. The rats on the unsupplemented homocystine diet (I) showed the expected weight loss, high liver fat content, and extensive kidney hemorrhage. Supplementation of this diet with choline (II) or substitution of methionine for homocystine (V) resulted in growth, lowered liver fat levels and minimal kidney pathology as anticipated from many previous studies. Growth with the methionine diet was somewhat superior to that with homocystine plus choline, but was considerably inferior to that obtained with the stock diet (VIII). This is no doubt to be attributed to deficiencies in amino acids other than methionine in arachin. Supplementation of the homocystine diet with diethyl choline did not permit growth, but a strong lipotropic action and a prevention of kidney hemorrhage were apparent. This is in accordance with previous observations.[1] However, the toxicity on growing rats reported for this compound[1] was not apparent in the present studies. The animals receiving both choline and diethyl choline (Group IV) did not differ significantly from those receiving choline alone.

The animals receiving ethionine (VI) lost weight more rapidly than did those receiving homocystine. This toxic effect of ethionine was partially prevented by addition of methionine. The toxic effect of ethionine on rats and the protection by methionine has already been reported by Dyer.[2] It is of utmost interest, however, that the accumulation of liver fat and the development of kidney hemorrhage did not develop on the ethionine diet.

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[3] Patterson, W. I., and du Vigneaud, V., *J. Biol. Chem.*, 1935, **111**, 393.

[4] Johns, C. O., and Jones, D. B., *J. Biol. Chem.*, 1916, **28**, 77.

[5] Engel, R. W., *J. Biol. Chem.*, 1942, **144**, 701.

A most outstanding result apparent from Table II is the fact that the total liver choline (or its ethyl analogues) is very markedly reduced in the animals on homocystine plus diethyl choline (Group III) and on ethionine (Group VI).

*Discussion.* It is of special interest that ethionine should show a lipotropic action and a protection against kidney hemorrhage, since it has no methyl groups and does not have a structure which can reasonably be considered to substitute for choline in phospholipids. It does not seem likely that the lipotropic and antihemorrhagic action of ethionine can be explained on the basis of a transethylation to ethanolamine to form an ethyl analogue of choline, since such choline analogues were not found in the livers of the animals of Group VI.

The possibility that diethyl choline exerts its lipotropic action by virtue of its structural relationship to choline and consequent incorporation into phospholipid would seem to be supported by the observation of McArthur,[6] who showed the presence of administered triethyl choline in the liver phospholipids of rats. However, in our experiments we found very much lower than normal amounts of choline or its analogues in the livers of animals fed homocystine plus diethyl choline (Group III).

It is therefore concluded that the action of ethionine and diethyl choline in preventing fatty livers and kidney hemorrhage of rats on a low choline and low methionine diet probably does not involve their incorporation in whole or in part into phospholipids.

*Summary.* The toxic actions of ethionine on the growth of rats and the protective action of methionine reported by Dyer[2] has been confirmed. It has been shown that ethionine prevents the accumulation of liver fat and the hemorrhagic kidney degeneration on low methionine, low choline diets. The diethyl analogue of choline also prevents the fatty livers and kidney lesions of rats on similar diets. The action of these compounds does not appear to involve their direct participation in phospholipid formation.

TABLE II.

Weight Changes, Liver Fat Content, Liver Choline Content and Kidney Hemorrhage in Rats.

| Group | Diet | No. of animals | Avg initial wt (g) | Avg food eaten per day (g) | Wt gain per day (g) | Liver fat % dry wt* | Liver choline† mg/g | Kidney damage |
|---|---|---|---|---|---|---|---|---|
| I | Homocystine | 14 | 59 | 6.2 | —1.0 | 41.0 ± 2.5 | 2.1 | ++++ |
| II | Homocystine + choline | 13 | 61 | 8.1 | 1.5 | 18.8 ± 1.4 | 2.8 | +— |
| III | Homocystine + diethylcholine | 10 | 57 | 5.0 | —0.7 | 8.4 ± 1.0 | 0.1 | +— |
| IV | Homocystine + choline + diethylcholine | 10 | 68 | 7.7 | 1.0 | 13.8 ± 1.1 | 2.9 | +— |
| V | Methionine | 25 | 63 | 9.4 | 1.6 | 14.3 ± 1.1 | 1.8 | +— |
| VI | Ethionine | 6 | 66 | 2.8 | —2.6 | 9.8 ± 1.4 | 0.2 | +— |
| VII | Ethionine + methionine | 5 | 67 | 7.2 | 1.1 | 23.4 ± 1.4 | 3.6 | +— |
| VIII | Stock | 10 | 59 | 11.0 | 3.3 | 7.4 ± 0.3 | 2.3 | — |

* Including the standard error of the mean.
† Including the monoethyl, diethyl, and triethyl analogues.

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[6] McArthur, C. S., *Science*, 1946, **104**, 222.

### 16675

## Intravenous Saline Tolerance in Mice.

Martin M. Winbury* and Phoebe J. Crittenden.†
*From the Merck Institute for Therapeutic Research, Rahway, N. J.*

In the course of investigating the comparative toxicity of certain sparingly soluble compounds in mice, it was necessary to inject as much as 5 cc per 20 g of body weight of hypotonic and isotonic solutions. In order to determine the effects of injecting such large volumes without the influence of any drug, the intravenous toxicity of sodium chloride solutions at different osmotic concentrations was investigated. In addition the rate of injection was varied to determine the maximum speed permissible for safe administration.

Male mice of the CF 1 strain, weighing from 19 to 30 g were used and all animals surviving the initial injection were observed one week for delayed deaths. The solutions were administered into one of the lateral veins of the tail at a given rate by means of a constant speed infusion pump.

In the first experiment physiological saline (0.85% NaCl, 0.15N) was administered until death occurred. At the rate of 0.9 cc per minute, an average of 6.9 ± 0.71[‡] cc per 20 g was found to be the fatal dose. Although increasing the rate of administration above 1 cc per minute (1 to 2.5 cc per min.) appeared to decrease the fatal dose to 5.9 ± 0.63[‡] per 20 g, the difference between the 2 sets of data is not significant. After establishing the fatal dose for physiological saline, a second experiment was carried out in which various concentrations of sodium chloride were injected at different rates until the animals received 5 cc per 20 g or less if death occurred. Accordingly the animals were divided into 5 groups receiving one of the following solutions: distilled water, 0.425%, 0.85%, 1.70%, or 2.55% sodium chloride at rates ranging from 0.2 to 4.0 cc per minute. As is shown in Table I, half physiological saline (0.425%, 0.073N) is the least toxic; 5 cc per 20 g was tolerated by all animals at a maximum rate of 3.50 cc per minute. Increasing the rate above this point results in death. As the salt concentration was increased, tolerance to 5 cc per 20 g was reduced. With physiological saline, this volume was tolerated at a maximum rate of 2.5 cc per minute, but in the case of distilled water, 1.70 or 2.55% sodium chloride, 5 cc per 20 g could not be administered safely, for some deaths occurred even at the slowest rates. It would hardly be practical to administer 5 cc per 20 g at rates below 0.2 cc per minute because of the time required for the injection.

Both physiological and half physiological saline are well tolerated under the circumstances of this experiment whereas the hypertonic solutions are not "safe" since deaths occurred even at the slowest rates of administration. Although the half physiological saline appears to be slightly less toxic than the physiological saline this difference is not statistically significant. Therefore, when large volumes (5 cc per 20 g body weight) are required in intravenous toxicity studies in mice, it would seem most logical to use solutions with an osmotic concentration between the range of 0.073N to 0.15N. This is equivalent to 0.425% to 0.85% NaCl. In our studies we adjusted the solutions of the sparingly soluble compounds to 0.073N and injected up to 5 cc per 20 g at a rate of 1.5 cc per minute.

*Summary.* Mice tolerated intravenous injections of 5 cc per 20 g body weight of half physiological and physiological saline at maximum rates of 3.5 and 2.5 cc per minute respectively. The average lethal dose for physiological saline given at a rate of 0.9 cc per minute is 6.9 cc per 20 g.

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[†] Present address: Dept. of Physiology, Goucher College, Towson 4, Md.

[‡] Standard error.

TABLE I.
Toxicity of Various Sodium Chloride Solutions Administered Intravenously at Different Rates.

| | Rate of administration cc/minute 4.0-3.5 | | 2.9-2.5 | | 2.4-2.0 | | 1.9-1.5 | | 1.4-1.0 | | 0.9-0.5 | | 0.5-0.2 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| % NaCl | cc/20 g | D* | cc/20 g | D* | cc/20 g | D* | cc/20 g | D* | cc/20 g | D* | cc/20 g | D* | cc/20 g | D* |
| 0 | 4.7† | 4/4 | 4.6† | 3/3 | 5.1 | 1/1 | 4.5† | 1/1 | 4.2† | 1/1 | 3.9† | 1/1 | 4.6† | 2/2 |
| 0.425 | 5 | 3/10 | 5 | 0/9 | 5 | 0/6 | 5 | 0/3 | 5 | 0/2 | 5 | 0/1 | | |
| | | | | | | | | | ''Safe'' range | | | | | |
| 0.85 | 5 | 4/6 | 5 | 2/8 | 5 | 0/3 | 5 | 0/4 | 6.4‡ | 0/3 | 5.0 | 0/6 | 5.0 | 0/1 |
| 1.70 | 3.9† | 1/1 | 4.0† | 2/2 | | | | | 4.2† | 1/1 | 5.0 | 1/3 | 5.0 | 1/3 |
| 2.55 | 2.2† | 1/1 | | | | | 1.8† | 1/1 | 2.6† | 1/1 | 2.9† | 3/3 | 5.1 | 2/2 |

* $D = \frac{\text{No. dead}}{\text{No. dosed}}$

† Death before 5 cc injected.

‡ Two of these animals received 7 cc per 20 g.

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## 16676

# Failure of Xanthopterin to Influence Hematopoiesis and Growth in Rats.*

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Tschesche and Wolf[1] reported that rats fed goats' milk develop an anemia which responds to xanthopterin. Simmons and Norris[2] and Simmons[3] observed that anemia in Chinook salmon responds to injections of xanthopterin. Totter and Day[4] reported that in rats with the deficiency induced by feeding a purified diet containing succinylsulfathiazole, administration of 20 μg of xanthopterin per day caused a leukocytosis and a marked weight gain, but in subsequent experiments they failed to confirm this observation. Wright and Welch,[5] Daft and Sebrell,[6] and Axelrod, *et al.*,[7] also failed to note any effect when xanthopterin was fed to rats deficient in pteroylglutamic acid (PGA). Totter and co-workers[8,9] reported that favorable hematopoietic responses occur when xanthopterin is given to vitamin M-deficient monkeys. In this laboratory, 2 macrocytically anemic swine have shown a submaximal hematopoietic response to large parenteral doses of xanthopterin (10 mg daily),[10] a phenomenon which is being investigated further.

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† Present address: Army Medical Department Research and Graduate School, Army Medical Center, Washington, D.C.

[1] Tschesche, R., and Wolf, H. G., *Ztsch. f. physiol. Chem.*, 1937, **248**, 34.

[2] Simmons, R. W., and Norris, E. R., *J. Biol. Chem.*, 1941, **140**, 679.

[3] Norris, E. R., and Simmons, R. W., *J. Biol. Chem.*, 1945, **158**, 449.

[4] Totter, J. R., and Day, P. L., *J. Biol. Chem.*, 1943, **147**, 257.

[5] Wright, L. D., and Welch, A. D., *Science*, 1943, **98**, 179.

[6] Daft, F. S., and Sebrell, W. H., *Public Health Rep.*, 1943, **58**, 1542.

[7] Axelrod, A. E., Gross, P., Bosse, M. D., and Livingle, K. L., *J. Biol. Chem.*, 1943, **148**, 721.

[8] Totter, J. R., Shukers, C. F., Kolson, J., Mims, V., and Day, P. L., *Fed. Proc.*, 1943, **2**, 72.

[9] Totter, J. R., Shukers, C. F., Kolson, J., Mims, V., and Day, P. L., *J. Biol. Chem.*, 1944, **152**, 147.

Cerecedo and his associates[11,12] found that xanthopterin, like PGA, when added to highly purified diets fed to mice or rats, causes a significant improvement in lactation performance. Fraenkel and Blewett[13] have reported that the growth of the larvae of the insect, *Ephestia kuehniella,* on an artificial diet which contains all the known factors of the vitamin B-complex in pure form, is markedly accelerated by the addition of PGA, and that xanthopterin, in concentrations about 1000-times greater, has a comparable effect.

Recently Norris and Majnarich[14,15] have studied the effects of xanthopterin in experiments conducted both *in vitro* and *in vivo.* Studies *in vitro* involved the use of cultures of bone marrow from rats and other animals. Xanthopterin in low concentrations was reported to cause an increase in the rate of red and white cell proliferation. In rats fed a purified diet containing sulfathiazole (1%), it was claimed that xanthopterin is hematopoietically active. They reported that xanthopterin *in vitro* and *in vivo,* is more effective than PGA, since it appeared to act in smaller doses and more rapidly. They concluded that bone marrow cannot use PGA directly in hematopoiesis while xanthopterin can be so utilized.

Findings with xanthopterin have been so variable, and the claims made by Norris and Majnarich are so remarkable, that in this study a further attempt has been made to determine whether xanthopterin can play a role in hematopoiesis and growth in rats. The data obtained do not support the conclusions drawn by Norris and Majnarich.

*Methods.* Weanling female albino rats[‡] were kept singly in cages with wire mesh floors. The basal diet had the following composition (in g per 100 g): purified casein, 20; glucose ("Cerelose"), 48.7; hydrogenated vegetable oil ("Primex"), 18; corn oil, containing vitamins A and D,[§] 2; salt mixture (U.S.P.X. No. 2), 2.8; accessory salts[‖] 0.2; cellulose ("Cellu-flour"), 5; choline chloride, 0.2; inositol, 0.1; thiamine hydrochloride and pyridoxine hydrochloride, each, 0.0005; riboflavin, 0.001; nicotinamide, 0.005; calcium pantothenate, 0.0055; ascorbic acid, 0.01; 2-methyl-1,4-naphthoquinone, 0.001; biotin, 0.00001.

*Group I.* Sixteen of the weanling rats were placed on the above basal diet with 1% *sulfathiazole* incorporated. At the end of 28 days, 3 rats were dead and 3 rats showed anemia without leukopenia. During the following 21 days, 5 more rats became anemic. In those rats that developed anemia during the latter period, leukopenia also was present. During this 21-day period, 3 rats died before developing an anemia of a level suitable for experiment; at the end of this period an erythrocyte level exceeding 5,000,000 per cm was found in 2 rats. Each of 3 rats (No. 78, No. 80 and No. 82) was given 100 μg of xanthopterin parenterally per day for 6 days; also one (No. 81) was given 200 μg per day, and another (No. 76) was given 400 μg per day, for 6 daily injections. Each of 5 rats (No. 69, No. 73, No. 80, No. 82, and No. 84) was given 50 μg of PGA daily for 6 injections; 2 of these rats (No. 80 and No. 82) previously had been treated with xanthopterin. The variable effects of xanthopterin and PGA are considered in the section entitled *Results.*

*Groups II and III.* Forty rats were placed on the basal diet in which 2% *succinylsulfathiazole* was incorporated. At the end of 28 days, when the rats showed marked im-

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[10] Pritchard, J. A., Welch, A. D., and Heinle, R. W., unpublished data.

[11] Mirone, L., and Cerecedo, L. R., *Arch. Biochem.,* 1948, **15**, 324.

[12] Sica, A. J., Allgeier, A. M., and Cerecedo, L. R., *Arch. Biochem.,* 1948, **18**, 119.

[13] Fraenkel, G., and Blewett, M., *Biochem. J.,* 1947, **41**, xviii.

[14] Norris, E. A., and Majnarich, J. J., *Am. J. Physiol.,* 1948, **152**, 175.

[15] Norris, E. A., and Majnarich, J. J., *Am. J. Physiol.,* 1948, **152**, 178.

[‡] Obtained from Blaine Biological Gardens, Media, Pa.

[§] Two grams of the corn oil preparation contained vitamin A, about 4500 units and vitamin D, about 560 units.

[‖] Accessory salts contained in 0.2 g (expressed in mg): KCl, 100; NaCl, 87.2; $FeSO_4$, 10; $MnSO_4$, 1; $ZnSO_4 \cdot H_2O$, 1; NaI, 0.3; NaF, 0.1; $CoCl_2 \cdot 6H_2O$, 0.01.

pairment of growth, leukocyte and hemoglobin levels were determined. Twelve severely leukopenic rats were assigned to group II. In these rats the effects of xanthopterin and of PGA on leukopoiesis and growth were studied. Each of 6 rats was injected with 50 $\mu$g of PGA daily for 5 days. Into the remaining 6 rats, xanthopterin was injected daily for 5 days; the daily dosage was 100 $\mu$g in one rat, 200 $\mu$g in 4 rats, and 400 $\mu$g in the sixth rat. Leukocyte counts and weighings were made at 3-day intervals. The positive effects of PGA and the lack of effect of xanthopterin are considered under *Results*.

The remaining 21 rats, of the group given the purified diet plus 2% succinylsulfathiazole, composed group III. In this group a study was made of the effects of xanthopterin and of PGA on hemoglobin synthesis, as well as on growth and leukopoiesis. Under these conditions, severe anemia failed to develop during a period of 28 days. However, an anemia was induced by means of hemorrhage, using a technic similar to that described by Kornberg *et al.*[16] Blood, in an amount equivalent to approximately 2% of the weight of the rat, was removed at each bleeding. Sufficiently low hemoglobin levels were produced by 2 to 4 bleedings of each rat, carried out at intervals of 2 days. Two rats died during the course of the bleeding, and 2 rats died 2 days following the last bleeding. Seventeen rats were available for experiment, of which 4 served as untreated controls. Each of 4 rats was given parenterally 50 $\mu$g of PGA daily for 5 days. Nine rats were given xanthopterin parenterally; 5 of the 9 were given 100 $\mu$g each, daily for 5 days, while 4 were given twice this dose. Weighings, leukocyte counts and hemoglobin measurements were done at 3-day intervals. Hemoglobin was determined as oxyhemoglobin using a Klett-Sumerson photoelectric colorimeter. The findings are presented in Figure 1 and are discussed under *Results*.

*Results. Group I* (*1% sulfathiazole*). In rats given a purified diet containing succinyl sulfathiazole, anemia is rarely seen. However, the use of sulfathiazole in a purified diet induced, during a 7-week period, an anemia in 8 of 10 surviving rats.

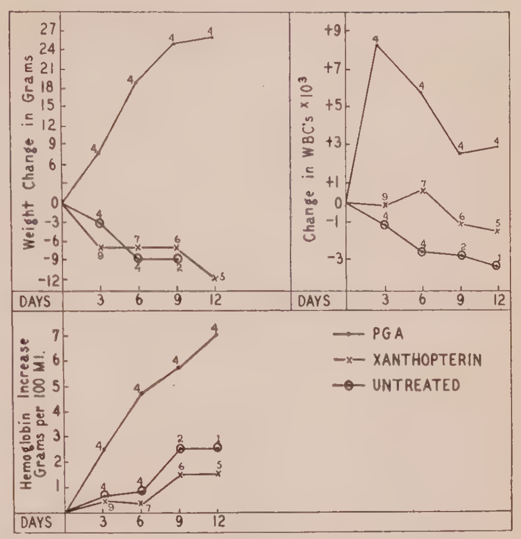

FIG. 1.

Changes in hemoglobin levels, leukocyte counts, and weight in rats fed a purified diet containing 2% succinylsulfathiazole and bled 2 to 4 times. One group received pteroylglutamic acid, one group received xanthopterin, and a third group served as the untreated control. The numbers at each point in the figure represent the number of surviving rats represented in the average.

The data presented here indicate clearly that a markedly variable *hemolytic anemia* has occurred in rats fed sulfathiazole in a purified diet. Reticulocytoses of 10 to 25% were noted in many of the anemic rats before therapy. Those rats in which anemia developed earlier did not show leukopenia. Therefore, leukopoiesis and erythropoiesis, as judged by reticulocytosis, certainly was occurring. It is evident that the anemia was due to factors other than one related directly to the lack of an essential hematopoietic factor or factors. The feces of many of the rats were observed to contain great amounts of bile pigments. One rat (No. 69), while receiving a total dose of 300 $\mu$g of PGA parenterally, showed a decrease in the number of circulating erythrocytes from 4.0 million to 1.7 million per cmm in 6 days, yet during this same period, the leukocyte count increased from 3,700 to 68,500 per cmm; on the sixth day, a recticulocytosis in excess of 80% was present. The marked anemia, with a marked leukocytosis and reticulocytosis, was maintained. The rat died on the twelfth day after the start of therapy. A similar fall in erythrocyte count was noted in a rat (No. 76) which received a total dose of 2400 μg of xanthopterin. The red blood cell level fell from 4.0 million to 2.9 million per cmm; a reticulocytosis of 20% was present on the sixth day. During this 6-day period, the leukocyte count increased from 4,900 to only 6,700 per cmm. The rat died 15 days after treatment with xanthopterin was started.

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[16] Kornberg, A., Tabor, H., and Sebrell, W. H., *Am. J. Physiol.*, 1944, **142**, 604.

Four rats (No. 78, 80, 81 and 82) showed a slight increase in the number of circulating erythrocytes after treatment with xanthopterin. During the 9 days following the start of xanthopterin therapy, an average increase in erythrocytes of 1.4 million per cmm occurred; the largest increase was 1.8 million (No. 80). The leukocyte counts fell in all 4 rats. No rat receiving xanthopterin showed a positive growth response.

In the 5 rats given PGA, variable effects on growth and circulating erythrocyte levels were obtained. A marked leukocytosis occurred in all PGA-treated rats. The largest increase in erythrocyte count was 3.3 million per cmm (No. 82). The small increase in erythrocyte counts that occurred in rats given xanthopterin, did not occur earlier than in those given PGA.

*Group II* (*2% succinylsulfathiazole*). The effect of xanthopterin and of PGA on growth and leukopoiesis were compared in leukopenic rats with arrested growth. Six rats given a daily dose of 50 μg of PGA parenterally for 5 days showed favorable leukocyte and growth responses during the 12 days of study. Six days after the start of therapy an average increase in white blood cells of 12,900 per cmm was noted. Six rats receiving 2 to 8 times as much xanthopterin parenterally showed an average increase of 1,300 per cmm. At the end of 12 days those rats given PGA all were alive and an average gain in weight of 21 g was observed. The xanthopterin-treated rats showed an average loss in weight of 11 g during this same period. One rat given xanthopterin died on the sixth day. No differences were observed in leukocyte and growth responses to different dose levels of xanthopterin. "Spectacled" eyes and ruffled fur developed in rats during the period of administration of xanthopterin. The lack of effect of xanthopterin on growth and leukopoiesis is similar to that previously observed.[5-7]

*Group III* (*2% succinylsulfathiazole plus hemorrhage*). It was felt that the study of the comparative effects of xanthopterin and PGA on erythropoiesis could be conducted more satisfactorily in deficient rats made anemic by hemorrhage than in rats with an anemia due to an active hemolytic process.

Definite hematopoietic and growth responses followed therapy with PGA. Little difference between the responses occurring in the xanthopterin-treated anemic group and in the untreated anemic control group was noted.

In the 4 rats given PGA, the average hemoglobin concentration at the start of therapy was 6.6 g per 100 ml of blood. During the 12 days after the start of therapy, an increase of 7.1 g to an average concentration of 13.7 g per 100 ml occurred. This was accompanied by an average growth response of 26 g. An average increase in leukocytes of 8,300 per cmm was noted on the third day. All the PGA-treated rats were alive at the end of the experiment.

Nine anemic rats were given xanthopterin in doses 2 to 4 times larger than the dose of PGA used. The average hemoglobin concentration at the start of therapy was 6.3 g per 100 ml of blood. The hemoglobin concentration at the end of 12 days in the 5 surviving rats was 8.1 g. No favorable leukopoietic response was noted. Loss of weight continued in spite of xanthopterin therapy. The 5 surviving rats lost an average of 12 g during the 12 days following the start of therapy. "Spectacled" eyes and ruffled fur developed in spite of the administration of the pterin. Three of the 5 surviving rats died during the 3 days following the last blood determinations. No significant difference was noted between those rats given 200 μg and those rats given 100 μg of xanthopterin per day.

Four anemic rats were not treated. Their average hemoglobin concentration was 8.2 g per 100 ml at the start of the observation period. The average hemoglobin level closely paralleled that of the xanthopterin-treated anemic rats during the 12-day observation period. Only 1 rat was alive at the end of 12 days. Weight and leukocyte changes also were similar to those noted in the xanthopterin-treated rats.

*Discussion.* Norris and Majnarich[15] have not indicated the mechanism of production of the anemia they observed in rats fed a purified diet containing sulfathiazole. Sulfathiazole has been reported by Richardson[17] to cause hemolytic anemia and the hemolytic anemia-producing properties of related sulfonamides have been studied by Latven and Welch.[18] That such a hemolytic process operated in the rats of Norris and Majnarich[15] is indicated by the production of an anemia within 22 days after the diet was started, and by the rapid fall in erythrocyte counts noted after the brief rise following therapy. Against a hemolytic process are the apparently very low reticulocyte levels which were reported by them; it would seem certain, however, that a measurable erythrocytic response following therapy in any type of severe anemia would produce greater reticulocyte responses than the 0.8 and 1% maximum values that they observed. It is suggested that the reticulocyte counts reported by Norris and Majnarich[15] are in error.

The effects shown here of PGA and xanthopterin in rats made anemic by feeding a purified diet containing sulfathiazole do not confirm the observations of Norris and Majnarich.[15] No positive leukocytic responses were noted after therapy with xanthopterin, although marked responses occurred after the administration of PGA. Doses of xanthopterin larger than the doses they used failed to produce the distinct increases in circulating erythrocytes that they recorded. Highly variable results also were obtained when PGA was given to those rats fed a purified diet containing sulfathiazole; however, those PGA-deficient rats made anemic by hemorrhage, and then given PGA, all showed nearly equal, marked erythropoietic responses.

*Summary.* 1. A purified diet containing sulfathiazole produced a hemolytic anemia of variable intensity which could not be completely reversed either by pteroylglutamic acid or by xanthopterin in any of the doses used.

2. Rats fed a purified diet containing succinylsulfathiazole developed leukopenia and an inhibition of growth which responded to PGA, but not to xanthopterin.

3. In similar rats fed a purified diet containing succinylsulfathiazole, but made anemic by repeated hemorrhage, PGA induced definite erythropoietic, leukopoietic and growth responses, while xanthopterin was inactive.

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[17] Richardson, A., *J. Pharmacol. and Exp. Therap.*, 1941, **72**, 99.

[18] Latven, A. R., and Welch, A. D., *J. Pharmacol. and Exp. Therap.*, 1944, **81**, 301.

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## 16677

# Stripping Film Technics for Histological Autoradiographs.

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*From the School of Medicine and Dentistry, Department of Radiation Biology, University of Rochester, Rochester, N. Y.*

There are now three general technics of autoradiography. The first method developed was that of placing the specimen in contact with a photographic plate, removing the specimen, and developing the plate. London[1] studied anatomical specimens in 1904 and Lacassagne and Lattes,[2] histological specimens

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[1] London, E. S., *Arc D'elec. Med.*, 1904, **12**, 363.

[2] Lacassagne, M. A., and Lattes, J. S., *Compt. rend. Acad. Sci.*, 1924, **178**, 488.

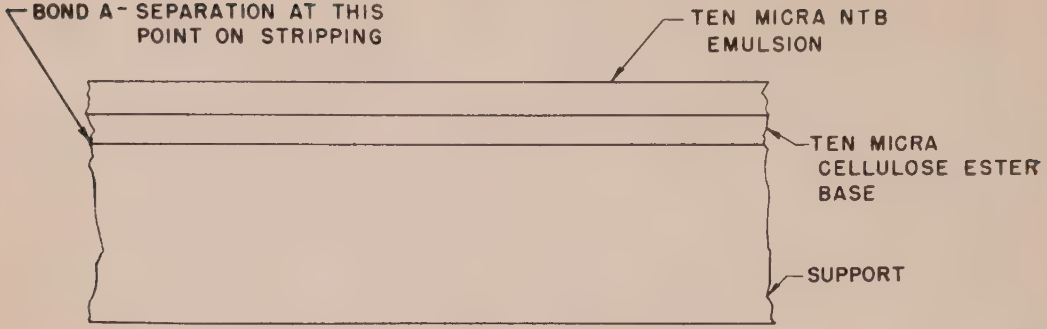
BOND A- SEPARATION AT THIS POINT ON STRIPPING
TEN MICRA NTB EMULSION
TEN MICRA CELLULOSE ESTER BASE
SUPPORT

FIG. 1.
Schematic illustration of Eastman NTB stripping film used for autoradiographs.

in 1924 in this manner. In recent years Bélanger and Leblond[3] developed the procedure of painting the tissue section with a photographic emulsion. Endicott[4] and Evans[5] independently worked out a third technic of permanently laying a tissue section on the emulsion of a plate or film. Each of these methods has its advantages and disadvantages, some of which have been discussed elsewhere.[6]

The use of stripping film has certain advantages. With the tissue mounted on the emulsion of the film stretched over a frame, back scattering is eliminated, and thus, in theory, resolution is increased. We have not as yet proved that this is of practical value. Another advantage is the ability to make autoradiographs of blood and bone marrow smears on microscope slides by the simple procedure of laying the film on the smear. With the histological specimen mounted on the photographically insensitive and water impervious base, it is separated by 10 $\mu$ from the autograph. This enables one to prevent harm to the tissue while developing and fixing and to eliminate staining of the emulsion. This separation of tissue and emulsion also enables one to identify histological bodies coincident with an alpha sunburst. Also, the separation opens the way to a semi-quantitative estimation of beta emitters by counting the grains which can be seen beneath the darkly stained nuclei because of the difference in the focal plane. And since the tissue is not in contact with the emulsion chemical fogging is eliminated, an application which will probably prove to be most valuable.

Beginning in the early fall of 1947, we have developed methods of using stripping film to achieve these advantages. During this work there appeared the paper of Pelc[7] on the use of stripping film.*

*Description of the film.* The stripping film was made in experimental quantities for this work by the research laboratory of the Eastman Kodak Company. Nuclear Track Emulsion B (NTB) was used as this had been found to be the most sensitive to beta particles for its grain size. It can also be used for alphagraphs. The sensitivity and fogging qualities of the emulsion on plates will be discussed in another paper. The stripping film comprises a thick cellulose ester support to one side of which has been bonded a thin film (approximately 10 $\mu$) of another cellulose ester which serves as the base for the emulsion. A diagrammatic sketch is shown in Fig. 1. The thicker cellulose ester serves merely as a rigid support for the manufacture and transport of the base-emulsion combination, the latter being the film which is stripped.

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[3] Bélanger, L. F., and Leblond, C. P., *Endocrinology*, 1946, **39**, 8.

[4] Endicott, K. M., and Yagoda, H., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 170.

[5] Evans, T. C., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 313.

[6] Evans, T. C., *Nucleonics*, 1948, **2**, 52.

* While this paper was in the process of declassification there appeared another paper on stripping film: "Radioautograph Technic with $C^{14}$," A. M. MacDonald, Jock Cobb, and A. K. Solomon, *Science*, 1948, **107**, 550.

[7] Pelc, S. R., *Nature*, 1947, **160**, 749.

The base, *i.e.*, the 10 $\mu$ cellulose ester film, is required to hold the low rigidity emulsion. This is especially true of the heavily loaded NTB emulsion with its low gelatin content. The rigidity of the emulsion has been further reduced in comparison to the ordinary emulsion by having been reduced in thickness to increase resolution of the betagraphs. Most of the satisfactory emulsions which we have used have been approximately ten micra although other thicknesses can be obtained.

*The background grain problem.* While the NTB emulsion on plates has an extremely low fog background, the same emulsion when stripped from the support may contain considerable fog, depending upon the stripping conditions. This appears as long streaks of single grain width, streaks of multiple grain width, and splotches. The streaks of single grain and multiple grain width are always on the surface and can be differentiated from nuclear tracks which dive at various angles into the emulsion. Some nuclear tracks do occur occasionally on the surface, but their length and grain density, as determined by those that do dive into the emulsion, differentiate them from the surface streaks.

However, the splotches cannot be differentiated from a random distribution of grains produced by beta particles. Therefore, if one wished to use stripping film for betagraphs, every precaution must be taken to prevent fog development. While we do not yet know how to eliminate all background grains, the following hints will aid in reducing them.

The usual precautions must be taken against light leakage and the presence of fogging chemicals in both the darkroom and dark storage boxes. For example, it is imperative that dark boxes, even though made of 1″ wood, be painted inside and out with black paint. Unpainted wood will transmit sufficient light to fog these emulsions. Obviously, one should not touch the emulsion with the hands. Abrasion fogging can be prevented by lifting the film out of the box instead of pulling it out edgewise and by preventing contact of any non-yielding object with the emulsion. It should be emphasized that the technics of ordinary amateur or scientific photography will not suffice for precise beta autoradiography. A few fog grains which would not greatly influence interpretation of a spectrographic plate or a photomicrograph of a histological section can lead to error in the interpretation of a betagraph under high magnification. In the latter there must either be no background fog or a known and constant quantity if one wishes even a semiquantitative estimate of the beta emitter in a histological section.

*Stripping.* The stripping of the base from the support is one of the greatest sources of fog. In the breaking of the bond between the base and the support, static charges are produced. If the film is stripped rapidly, these charges produce a light visible to the dark-adapted eye. This, though small in amount, will fog the emulsion as can be determined by examination under the microscope with a 1.8 mm objective. If the film is stripped slowly so that no light is observed, the fog is reduced but not eliminated. Even with this precaution, however, the static charges are retained by the film. Apparently the charges either produce latent images directly or indirectly by unperceived photons. The charges can be reduced by stripping in a humid atmosphere.

The technic used in our laboratory is as follows: A section of the film of the required size is protected in some suitable manner with a light opaque shield in addition to the usual darkroom precautions. It can be wrapped in black paper if care is taken to prevent the stiff paper from scratching the emulsion. Another method is to use a dark box with a slot in one side, the slot being covered with velvet. In either case about 1 cm is exposed. With the exposed end on the table top, emulsion side down, cut deeply but not completely through the support. (The position of the emulsion to be used for the autograph will be some distance removed from this point and protected from radiation by the shield.) Break only the support at the cut, *i.e.*, do not break the support and the base. On the break, photons are generated but the shield protects the emulsion. After the break the shield is removed and the base is stripped slowly while blowing the humid breath onto the point of bondage to discharge the static electricity. As the base-emulsion combination is stripped it should make as small an angle with the support as possible to avoid mechanical fogging due to creasing of the emulsion.

*Preparation of autograph with the tissue on the emulsion.* Autographs can of course be made by placing the tissue either on the emulsion or on the cellulose ester base. In the latter case, some resolution is lost because of the spatial separation of the tissue from the emulsion. For the highest resolution, the tissue is placed on the emulsion, thus decreasing to a minimum the distance between the origin of the ionizing particles and the emulsion; and at the same time the stripping film on a frame eliminates backscattering.

A 2″ x 6″ section of the stripped film is floated on water, the emulsion side down. It is then transferred to a Lucite frame 2″ x 6″ x ⅛″ by placing the frame in the water beneath the film. In the center of the frame is a rectangular opening 1″ x 3″ which is covered with the film, exposing the emulsion inside the ⅛″ well and the cellulose base on the other side. The tissue is placed in this area on either side.

The tissue section has been prepared before entering the darkroom as follows: One or more sections from a ribbon of paraffin-imbedded tissue is floated on water in a petri dish on a hot plate.[5] The water is warmed until the wrinkles have disappeared. This is now picked up by inserting a section lift under the edge of the paraffin and lifting it from the water while the remaining portion of the section hangs from the edge. The angle at which the section lift is pulled out of the water as illustrated in Fig. 2 is necessary to enable the tissue to hang freely. This manipulation is facilitated by drilling a large number of holes in the section lift to permit the water to drain from under the section. If the water drains over the edge of the lift, the current will carry the tissue section back into the petri dish.

The free end of the tissue section is placed on the wet emulsion immediately after the film is placed on the Lucite frame. With a horizontal motion the tissue is freed from the section lift and laid flat on the film. Any excess water is permitted to drain off or is picked up by a small hand syringe. The preparation is placed in a lead lined dark box in the refrigerator for exposure.

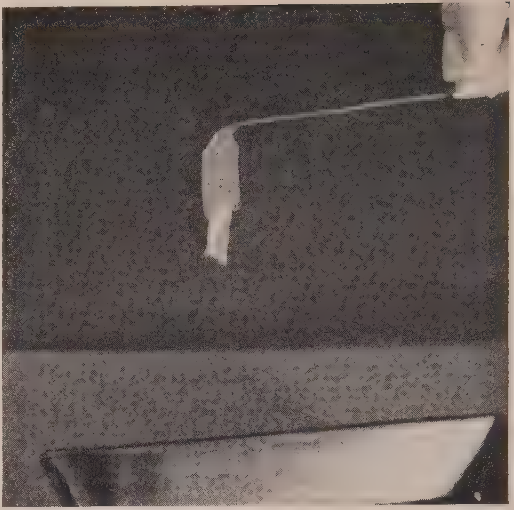

FIG. 2.

Removing a tissue section from water with a section lift, the perforated spoon held vertically to prevent the tissue from curling around the edge. The bottom of the tissue is touched to the photographic emulsion with the lift in the same vertical position after which the spoon is backed away horizontally from the tissue, permitting it to lie on the surface.

As the film dries it becomes taut and smooth over the 1″ and 3″ well and the gelatin of the emulsion bonds the film to the Lucite frame. Occasionally after long exposure the film becomes loose at the end of the frame and curls. Paper clamps can be used to prevent this, but they should not be put in place until after the film has dried. If placed on earlier, the contraction of the gelatin in drying may break the film.

The paraffin is removed in the usual manner before development[5] which is accomplished by passing the frame, film, and tissue as a single unit through the solutions. After development in D-19 at 20°C for 2 minutes, fixing in 30% hypo and washing for about 10 minutes each, the 1″ x 3″ film is cut out and placed on a microscope slide, the emulsion side up. When thoroughly dry the cellulose ester base usually adheres to the glass. If it

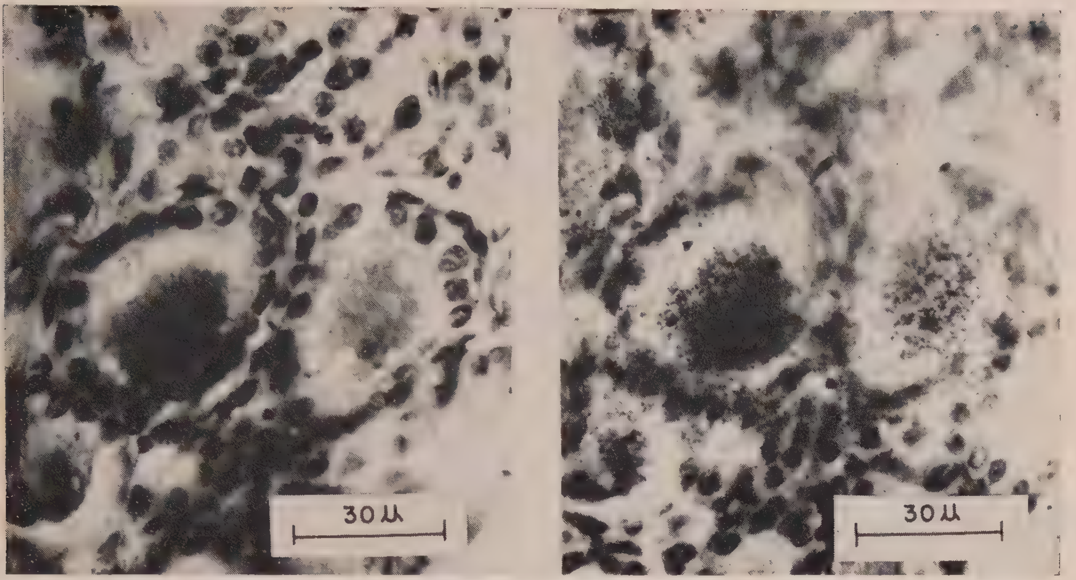

Fig. 3.
a. (left) Tissue level. b. (right) Grain level.
Photomicrographs of a thyroid tissue-autograph. A 7 μ section containing $I^{131}$ from a rat sacrificed 24 hours after injection of 500 μc, exposed 17 hours on NTB stripping film.

fails to bond, the glass can be painted with egg albumin or Kodak Stripping Film Cement. The excess film can now be cut away from the tissue and autograph area if desired. The entire unstained tissue and film can be mounted in Clarite, Permount, etc., and examined with the dark phase microscope.

However, since the developing and fixing solution slightly changes the refractive index differential of the histological bodies, *i.e.*, clears the tissue optically, the phase examination does not always give the best results. In this case the tissue and emulsion after having been placed on the slide can be stained in the usual manner. Some stain is taken up by the gelatin of the emulsion, but the gelatin content in ten micra of heavily loaded emulsion is low and the consequent uptake of hematoxylin and eosin is low in comparison with 7 μ of tissue. The background stain is insignificant and color contrast good at high magnification.

Fig. 3 shows a thyroid autograph taken with stripping film. The tissue was placed on the emulsion and later stained with hematoxylin and eosin. Fig. 3-a shows the tissue focal plane, originally photographed with a 1.8 mm objective and a 10x occular. The grain focal plane is shown in photograph 3-b. These illustrate a difficulty which, while it is not impossible to overcome, requires careful handling of the film. It will be seen that the entire field of each is not in good focus. This is due to uneven laying of the film on the glass slide.

*Preparation of the autograph with the tissue on the cellulose ester base.* If it is desired to eliminate all staining of the emulsion, the tissue can be placed on the cellulose ester base. In this procedure the base is painted with egg albumin to seal the tissue and prevent it from coming off in the developing, fixing, or staining solutions. However, as one or 2 days, is required for complete drying of the albumin, we have found it expedient in exposures under 2 days to develop and fix by placing the solutions in the ⅛″ well of the Lucite frame, removing them by tipping to one side and draining. This prevents the washing off of the tisue. The preparation is dried well before placing in the staining solutions.

Since in this method the paraffin is not removed before development, the tissue is protected and clearing is prevented. After development the tissue-emulsion area is cut out as described above and placed on a microscope slide, emulsion side down. The tissue is now deparaffinated in xylol and taken through the alcohol-water solutions to water and then the edges of the film sealed by dipping in molten paraffin. The tissue is then stained. The paraffin around the edges of the film is removed and the tissue-film combination mounted as described previously.

The emulsion which is protected by the aqueous-impervious paraffin and the cellulose ester is not stained, thus giving a high color differential between the tissue and the gelatin. The phase microscope can also be used without staining. The tissue intensity differentials as seen by the phase microscope have not been reduced by partial clearing, since the tissue has been protected in the developing and fixing solutions by the paraffin.

*Stripping film for blood smears.* The ideal blood smear for an autoradiograph has the cells separated by several micra so that the autograph of the beta emitters of one cell will not be confused with those of adjoining cells. It is difficult to make such a smear on an emulsion since the gelatin imbibes the water so quickly that the cells will not flow easily.

A thin smear is made on a microscope slide in the usual manner, stained for bright field illumination or left unstained for phase examination. In either case it is fixed for about 3 minutes with absolute methyl alcohol. After drying thoroughly, it is flooded with water and a piece of the film, emulsion side up, is floated over the smear. The water is then drawn out from under the film with filter paper, permitting the film to settle uniformly and without wrinkles over the smear. Since the fixing solution destroys the stain properties of the leukocytes, the procedure of paraffin dipping the edges is used to protect the smear during the developing and fixing steps. After washing and drying the paraffin is cut away with a razor blade and the smear, covered with the film, is mounted under a glass slip.

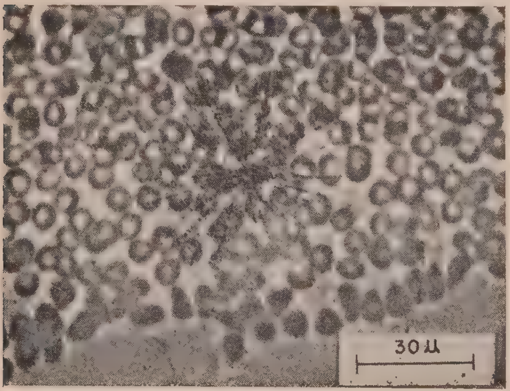

FIG. 4.
Sunburst of alpha tracks centered on a red cell.

Fig. 4 shows a concentration of a source of alpha particles coincident with one erythrocyte as evidenced by the center of an alpha track sunburst. This is a dark contrast phase photomicrograph of an unstained smear.

It should not be inferred from the photomicrograph that the alpha emitter is attached to the cell. It cannot yet be definitely stated whether the element concentrate is associated with the red cell in the blood stream or whether it has settled upon the cell in the smearing procedure. Some concentrations of alpha tracks have been found unassociated with cells. The exact nature of this phenomenon is being investigated and will be reported at another time.

Two types of fog patterns interfering with interpretation can be pointed out in this photomicrograph. In the lower right-hand corner of the photograph is a fog streak. It is obviously different from the straight alpha tracks of one grain width. Neither can it be a beta track as it is too dense and straight. Nor does it fit the description of other nuclear particle tracks.[8] This type of streak appears in the NTB stripping film and not in the NTB plate. We suggest that they originate in the stripping operation. In addition to the streaks the random fog grains should be noted. While these do not detract from the interpretation of an alphagraph, they do detract from that of a betagraph.

*Stripping film to permit identification of histological bodies above sunbursts.* Fig. 5-a and 5-b show alphagraphs which have been made by placing a liver section containing

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[8] Powell, C. F., Occhialini, G. P. S., Nuclear Physics in Photographs, Oxford at the Clarendon Press, 1947.

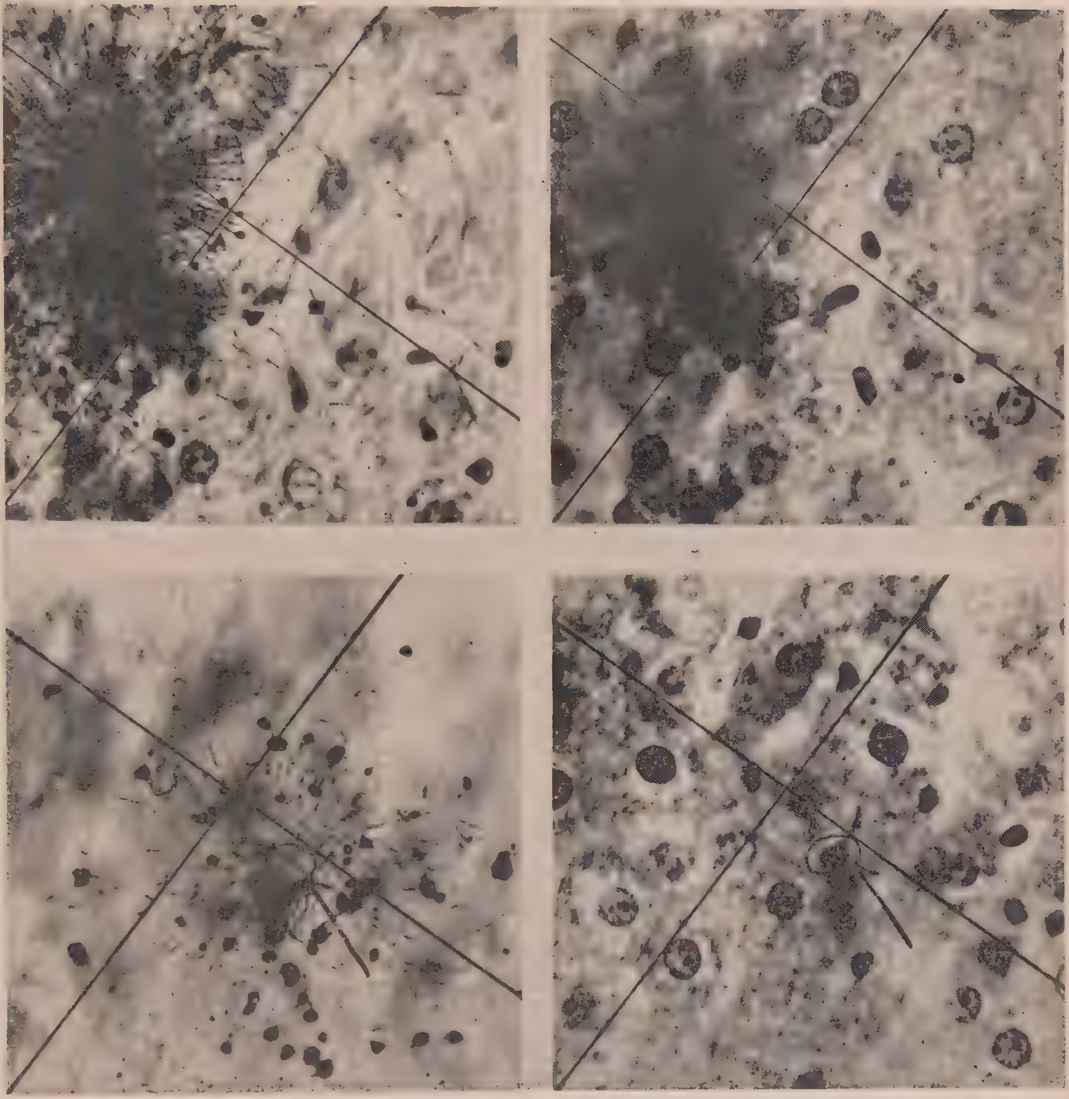

Fig. 5.

a (top left); b (top right); c (lower left); d (lower right).

Illustration of stripping film technic in identifying a histological body of rat liver containing an alpha emitter. In a and b the tissue section is on the emulsion of an NTA plate; a, track level; b, tissue level. In c and d the tissue is on the cellulose ester base of NTB stripping film; c, end of tracks in emulsion showing center of burst under a von Kupffer cell indicated in d.

an alpha emitter directly on an NTA (Nuclear Track A) plate, the former being a photograph of tracks in the emulsion, *i.e.*, just below the tissue-emulsion interface, and the latter is of the tissue directly above the tracks. Because of the high concentration of silver grains in the center of the sunburst there is insufficient illumination from below to permit observation or photography of the histological body directly above. Since this body presumably contains the collection of the radioactive element it is of interest to identify it.

When another section from the same liver specimen was placed on the cellulose ester support of a stripped film, photographs 5-c and 5-d were obtained. Only the low energy ends of the tracks are seen in 5-c, the concentration of silver grains being prevented by the insensitive layer of cellulose ester. Fig. 5-d shows sufficient illumination to observe and photograph the histological body directly above the center of the alpha track ends. This body (circled) appears to be a von Kupffer cell. This is a tentative conclusion awaiting further checking.

*Stripping film to permit estimating relative amounts of beta emitters in tissue.* The estimation of relative quantities of an alpha emitter is relatively easy since an alpha track representing a single disintegrated atom is a definite grain pattern and can easily be recognized. If the geometry and exposure time and half-life are known, a fair estimate of the concentration of an alpha-emitting element can be made. Certain precautions have to be taken. For example, the exposure must not be so long as to give a sunburst density as shown in Fig. 5-a in which the tracks cannot be counted. Also, if an optically dense histological body should override an alpha track, its chances of detection would be diminished.

These difficulties are magnified for the estimation of a beta emitter since no recognizable pattern of grains is produced. Instead, single grains must be counted. In the case of the thyroid, for example, where the colloid is reasonably transparent, the grains underneath the colloid can be easily distinguished one from the other. However, when one attempts to count the grains beneath the follicular epithelial cells, the grains are obscured by the optical density of the stained cells and their nuclei especially. This is illustrated in Fig. 3..

Stripping film offers an escape from this impassé. The tissue is mounted on the cellulose ester, 10 μ thick, instead of on the emulsion. Examining with a 1.8 mm objective having a field depth of about 0.5 μ and focusing the microscope 10 μ beneath a section to observe the grains, the optically dense bodies of the tissue are sufficiently out of focus and thereby transparent to permit counting the grains immediately beneath them. However, this advantage is not gained without some loss in resolution due to the 10 μ separation of the tissue from the emulsion. But this is a small loss compared with the gain in resolution of the NTB emulsion over that of medium lantern slide emulsions.

An additional advantage of the stripping film technic is a method of laying a uniformly thin emulsion layer close to a single cell or tissue. Either the emulsion or the 10 μ base can be placed in contact with the specimen. The latter differs from the emulsion-painting technic of Bélanger and Leblond[3] in that the emulsion is separated from the tissue by the thickness of the base. The stripping film has the advantage over the painting technic in that the base and emulsion are standardized at the factory by those experienced in the art of making photographic emulsions.

Another advantage of the stripping film is its use in the prevention of chemical fogging. As emulsions become more sensitive to beta particles we will be able to detect smaller amounts of beta emitters in a tissue section. However, as the technic becomes more sensitive, the problem of eliminating fogging due to various naturally occurring chemicals in the tissue looms large. The mounting of sections on the cellulose ester base instead of directly on the emulsion will eliminate this difficulty.

*Summary.* An autoradiographic technic, using stripping film, is described by which back scattering, emulsion staining, chemical fogging, and photographic-developer damage to the tissue are eliminated; blood smear autographs can be made; histological bodies above intense collection of grain can be identified; and grain counting for quantitation is permitted.

We appreciate the valuable assistance of Mr. Robert Hay in the preparation of the final photographs for this paper.

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16678

# Comparison of the Preputial Glands in the Alexandrine, The Wild, and the Domestic Norway Rat.*

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*From the Psychobiological Laboratory, Johns Hopkins Medical School, Baltimore, Md.*

During routine autopsies on wild Alexandrine and wild Norway rats, it appeared that the preputial glands of these wild rats were much larger than those found in the common domestic Norway rats. To check these impressions, the weights of the preputial glands were determined in the 3 forms for all sized rats from the smallest to the largest individuals.

*Material and methods.* For this study 78 domestic Norway, 121 wild Norway, and 90 Alexandrine rats were used.

The domestic Norways were obtained chiefly from our colony. These are descendants of Wistar rats established here over 20 years ago. The wild Norways were trapped in Baltimore streets and alleys and on surrounding farms by the Rodent Ecology Division of the Johns Hopkins School of Hygiene; the Alexandrines came from ships in the Baltimore harbor, where they had been killed by fumigation.‡

The domestic and wild Norways were killed with ether and autopsied immediately. The Alexandrines were not available for autopsy until 3 to 6 hours after they had been killed by fumigation.

At autopsy the rats were weighed; the perputial glands were removed and weighed on a torsion balance to the nearest 0.1 mg. In every case when sections were made, the glands were taken from recently killed animals and fixed in Bouin's solution with a minimum of handling.

*Results. Gross anatomy*: Fig. 1 shows a photograph of typical pairs of preputial glands taken from 3 female animals, a domestic Norway, a wild Norway, and a wild Alexandrine, all having about the same body weight. The glands from the wild Alexandrine are several times larger than those from the wild Norway and many times larger than those from the domestic Norway.

These glands are flattened, pear-shaped structures that lie between the skin and the lower abdominal musculature, one on either side of the penis or clitoris. Each gland terminates in a duct that emerges on the surface of the skin in a papilla-like opening. In the male, when the penis is fully retracted and thus ensheathed in the prepuce, the openings in the prepuce are located at the very end of the penis, whereas when the penis is fully erected and thus freed from the prepuce, the openings are located on the two sides at the base of the penis. In the female, the ducts emerge at the homologous points with relation to the clitoris.

A slight pressure exerted on the glands causes droplets of secretion to appear on the surface of the skin. These droplets help to locate the openings. In non-albinos, the presence of pigmentation in the walls of the ducts further helps to locate the openings.

*Microscopic anatomy*: A dense connective tissue capsule encloses the glandular parenchyma, which is composed of large, flat, polyhedral cells grouped into rounded acini and characterized by vesicular, pale-staining nuclei. Dissolution of the epithelial cells forms a secretion that appears as a granular eosinophilic mass within the numerous branched ducts.

Large wild Norway rats frequently have atypical glands in which numerous large cysts mar the usual smooth glandular surface (Fig. 2A). Such glands are found to consist of

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† Now at the University of Texas, Medical Branch, Galveston, Texas.

‡ Dr. M. F. Haralson, until recently in charge of the Quarantine Station of the City of Baltimore, kindly supplied these rats.

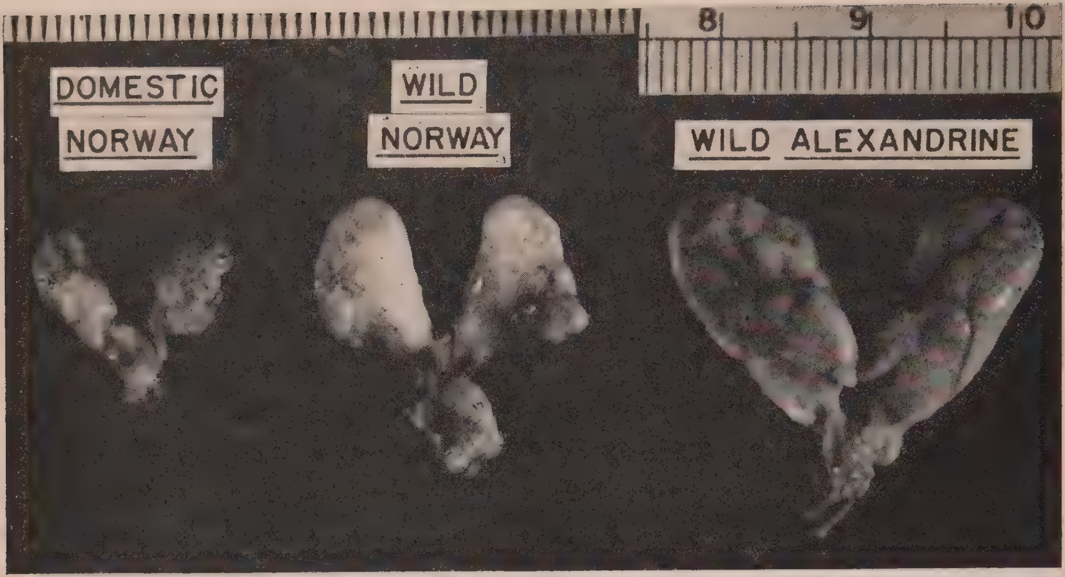


FIG. 1.
Photograph of the 3 pairs of preputial glands from a domestic Norway, a wild Norway, and a wild Alexandrine rat. Each rat weighed 250 g. (Scale—centimeters).

numerous dilated ducts, separated by strands of connective tissue. Many of these ducts show hyperkeratosis. Such cysts are generally associated with a chronic inflammatory reaction. It is not clear whether the inflammation causes, accompanies or results from the epithelial cysts.

Apart from this abnormality, routine staining with hematoxylin and eosin does not reveal any essential difference in glandular structure between the 3 types of rats. Figs. 2B and C are photomicrographs which show the typical structure of the preputial glands in Alexandrine and domestic Norway rats.

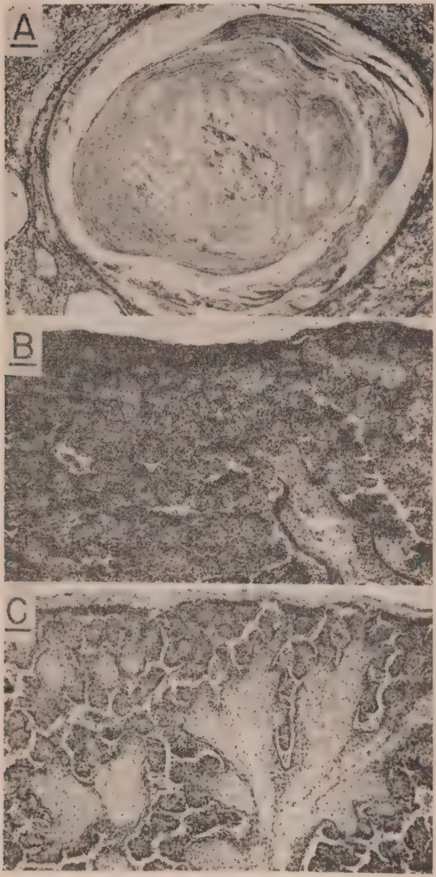
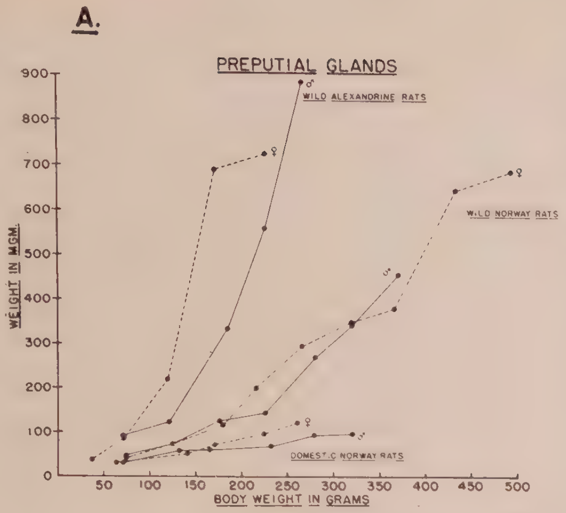


FIG. 2-A.
Low power view (×100) of a section from a preputial gland from a wild Norway rat. The center of the field is occupied by a large cystic dilatation containing a typical laminated epithelial "pearl" in the center of which degenerating acini may be seen.

FIG. 2-B.
Low power view (×100) of a section from the preputial gland of a domestic Norway rat. A branch of one of the ducts extends upwards from the lower right hand corner.

FIG. 2-C.
Low power view (×100) of a section of the preputial gland from a wild Alexandrine rat. A large multibranched duct is the most prominent feature.



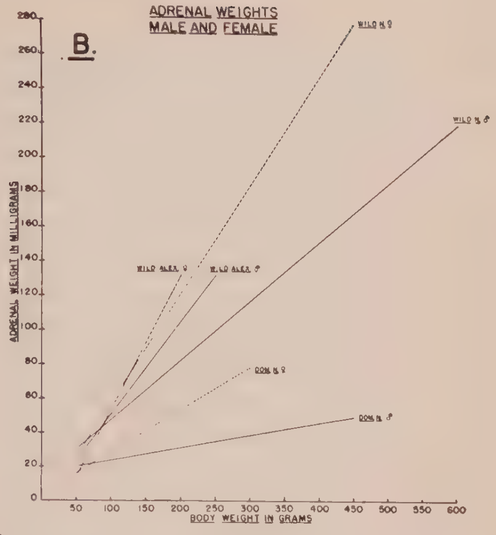


FIG. 3-A.

Graphs showing the relationship between the weights of the preputial glands and body weight in the 3 forms of rats: wild Alexandrines (45♂, 45♀); wild Norways (61♂, 60♀); domestic Norways (39♂, 39♀).

FIG. 3-B.

Graphs showing the relationship between the weights of the adrenals and body weight in the 3 forms of rats. (Data taken from Rogers and Richter).

*Physical characteristics of preputial secretion*: A considerable quantity (0.1-0.3 cc) of the sebaceous secretion usually may be expressed from the ducts of the glands by gentle manipulation in the mature wild Norway and the Alexandrine rats. It is seldom possible, however, to obtain as much from the glands of the domestic rat. In many rats the secretion is a viscous yellow mass; in some it may be quite liquid. In the former case it emerges in ribbon form, whereas in the latter droplets are formed.

*Growth and size of preputial glands*: Fig. 3A shows the relation between the weight of the preputial glands (in mg) and body weight. The lines connect points representing average values obtained in the various body weight classes.

a) *Wild Norways.* The weights of the preputial glands in female rats increased from 30 mg at an average body weight of 75 g to 695 mg at 500 g, or a 23-fold increase. The gland weights of the males increased at much the same rate.

b) *Domestic Norways.* The glands weighed much less in all body-weight classes of the domestic rats than in the wild forms, and their size increased at a much less rapid rate. The weights of the glands of female rats increased from 25 mg at an average body weight of 60 g to 115 mg at 350 g, or a 4.5-fold increase. Over the same weight range the glands of the wild Norways increased 11-fold. In general, female rats had larger preputial glands than males of the same size.

c) *Alexandrine rats.* At body weights of 250 g, the glands averaged 760 mg for Alexandrines, 200 mg for wild Norways, and 90 mg for domestic Norways. Thus the glands of Alexandrines weighed about 4 times as much as the glands of wild Norways and about 8.5 times as much as those of domestic Norways.

A comparison of the heaviest glands found at any time in each species further brings out the species differences: 143 mg in the domestic rat, 984 mg in the wild Norway, and 1200 mg in the wild Alexandrine.

The sharp increase that occurs in the size of the glands in the wild Alexandrine rat at body weights between 75 and 125 g, and in the wild Norways between 175 and 250 g, may coincide with the onset of puberty in the two species.

In the domestic Norway, which always has small glands, the variations from the mean for any weight group are small, whereas in the other two forms which have large glands, there is much more scattering, especially in the largest animals.

While comparing the size of the preputial glands in the three forms, one should bear in mind that the Alexandrine rat is normally a smaller animal than the Norway. A 250 g Alexandrine is an old adult of maximal size, but a 250 g wild Norway is barely an adult. If a comparison could be made of the relationship between preputial gland size and age, the difference between Alexandrine and wild Norway rats probably would not be as great as indicated by the present method. Unfortunately it is not possible to determine the age of trapped wild rats accurately, and laboratory raised Alexandrines of known age have not been available.

The preputial glands of both wild Norway rats and Alexandrine rats attain a greater size than has ever been produced experimentally with any form of treatment in the domestic Norway. Glands weighing about 300 mg have been reported in domestic rats following treatment either with potent adrenocorticotrophic extracts of the hypophysis (Noble and Collip,[1]) or androgens (Korenchevsky, *et al.*,[2-4]) but these are of moderate size compared to those found normally in adult Alexandrine or wild Norway rats.

*Cross breeding*: The offspring of both sexes from a cross between a wild Norway male and a domestic Norway female were used to determine the effects of cross breeding on the size of the preputial glands. Table I shows the relationship between the size of the preputial glands and body weight in the offspring of this cross as compared with wild Norway and domestic Norway rats of equal

TABLE I.

Weights of Adrenal and Preputial Glands in Wild and Domestic Norway Rats and in First Generation Crosses.

| Type | No. of females | Body wt | Preputial glands | Adrenals | No. of males | Body wt | Preputial glands | Adrenals |
|---|---|---|---|---|---|---|---|---|
| | | g | mg | mg | | g | mg | mg |
| Wild Norway | 7 | 215<br>(200-245) | 224<br>(144-335) | 90.1<br>(58.3-120.0) | 6 | 358<br>(330-370) | 426<br>(243-708) | 103.6<br>(74.5-158.0) |
| Wild x Domestic ($F_1$) | 7 | 216<br>(205-229) | 111<br>(90-136) | 66.5<br>(47.1-80.1) | 6 | 355<br>(327-369) | 126<br>(94-168) | 46.8<br>(45.0-48.2) |
| Domestic Norway | 7 | 220<br>(212-224) | 108<br>(72-127) | 52.9<br>(41.3-67.6) | 4 | 355<br>(352-359) | 109<br>(100-115) | 27.8<br>(20.6-35.3) |

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[1] Noble, R. L., and Collip, J. B., *Endocrinology*, 1941, **29**, 934, 943.

[2] Korenchevsky, V., Dennison, M., and Simpson, S. L., *Biochem. J.*, 1935, **29**, 2534.

[3] Korenchevsky, V., Dennison, M., and Eldridge, M., *Biochem. J.*, 1937, **31**, 467, 780.

[4] Korenchevsky, V., Hall, K., and Burbank, R., *Biochem. J.*, 1939, **33**, 213, 372.

size. In the cross-bred offspring, the glands are markedly smaller than those in wild Norway rats and only slightly larger than those in domestic Norways.

*Relation of preputial glands to adrenals*: Fig. 3B gives the average adrenal growth curves obtained for 100 (50 ♂ and 50 ♀) each of wild Alexandrine, wild Norway, and domestic Norway rats (data redrawn from Rogers and Richter[5]). The ordinates give the combined weights of both adrenals in milligrams; and abscissas, body weight in grams. Like the preputial glands, the adrenals of the wild rats are much larger than those of the domestic rats, and in each form the females have the larger glands. Unlike the preputial glands, however, the adrenals are only slightly larger in the wild Alexandrines than in the wild Norways.

In crosses between wild and domestic Norway rats the adrenals, like the preputial glands, are only slightly larger than in the domestic forms.

*Discussion.* The common laboratory Norway rat has undergone great changes in behavior during the course of domestication. It is tame, tractable and trusting; the wild rat is fierce, intractable, and suspicious. Some of the behavior changes can be correlated with anatomical modifications, such as the decrease in size of the adrenal glands (Rogers and Richter,[5]) or the decrease in the number of taste papillae on the surface of the tongue (Fish and Richter[6]). No such correlation can be readily detected between behavior and the recession of the preputial glands. The lack of any definite knowledge about the function of these glands makes a correlation difficult.

Since the ducts in males are located on the penis, it is possible that this highly sebaceous secretion may serve a lubricating function during copulation (Schaffer[7]). It seems unlikely, however, that it serves such a function in females, since in them the ducts do not end near the vaginal opening.

The secretion, through its odor (Noble and Collip[1]), might play a part in sex attraction. The fact that domestic rats have lived in cages for such a long time may be related in some way to the atrophy of these glands. Mates are provided for domestic rats, so that they no longer have to depend on finding their mates by scent.

The larger size of the preputial glands in the wild Alexandrines remains unexplained. The Alexandrine rats live more in ceilings, where any scent left would not be destroyed by rains, etc., as it is on the ground. Perhaps scent plays a larger part in their life than in that of the wild Norways.

*Summary.* 1. The domestic Norway rat, the animal commonly used in laboratories throughout the world, has much smaller preputial glands than does its wild ancestor.

2. The preputial glands are definitely larger in the wild Alexandrine than in the wild Norway.

3. In all 3 forms, the glands are larger in females than in males.

4. The preputial glands and the adrenals seem to show the same growth relationships in the three forms.

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[6] Fish, H. S., and Richter, C. P., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 352.

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# Effect of N,N-Dibenzyl-β-Chloroethylamine Hydrochloride (Dibenamine) on Autonomic Functions and Catatonia in Schizophrenic Subjects.*

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The schizophrenic reaction, catatonic type,[1] (dementia praecox, catatonic[2]) is characterized by conspicuous abnormalities of motor behavior. Patients with this disorder frequently also show signs of imbalance of the autonomic nervous system. Pupillary changes, vasomotor disturbances, increased salivation, scanty or increased urine, constipation, and cardiovascular irregularities may occur.[3] Gellhorn concluded that studies of autonomic functions in schizophrenic patients demonstrate "not only a decreased reactivity of the sympathico-adrenal system, but also a relative preponderance of the vago-insulin system."[4] Nevertheless, mydriasis, delayed gastric and colonic emptying,[5] cold extremities, and hyperhidrosis indicate elements of increased sympathetic activity.

The object of this investigation was to study the effect of chemical blockade of sympathetic function in patients with schizophrenic reactions, catatonic type. The drug used was Dibenamine (N,N-dibenzyl-β-chloroethylamine hydrochloride),† a synthetic tertiary amine having the specific, powerful and prolonged action of blocking excitatory adrenergic activity.[6]

In October 1947, as a preliminary procedure, Dibenamine was given to 2 catatonic patients at the New Jersey State Hospital at Greystone Park. Definite changes in autonomic functions and transient improvement in the catatonia were observed. Controlled studies were therefore arranged. The present report concerns our investigation of a group of catatonic subjects in whom certain autonomic functions were evaluated and neurologic and psychiatric examinations were made before and after administration of saline, sodium amytal and Dibenamine infusions.

*Methods and Materials.* Nine subjects, diagnosed as having schizophrenic reactions, catatonic type, were selected from the general wards at the Veterans Administration Hospital, Lyons, N. J. Patients with marked impairment of spontaneous activity and poor responsiveness to stimulation were sought. The cases with the severest stigmata of catatonia were chosen. The ages of the subjects ranged from 22 to 38 years, and their psychoses had been present from 3 to 7 years. Subject 4 was the only one who showed any recent tendency to have spontaneous remissions. Most of the patients had received electric and/or insulin shock therapy, with which varying degrees of transient benefit had been noted. No shock treatment was given to any of the group for at least 10 weeks prior to the beginning of this project, and no treatment other than the drugs being tested was given until after this work was completed. All subjects had received the routine hospital studies at the time of admission. Recent additional tests, including chest x-ray, E.C.G., C.B.C. and urinalysis, were within normal limits.

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* Published with permission of the Chief Medical Director, Department of Medicine and Surgery, Veterans Administration, who assumes no responsibility for the opinions expressed or the conclusions drawn by the authors.

[1] Nomenclature of Psychiatric Disorders and Reactions, Vet. Adm. T.B. 10A-78, Oct. 1, 1947.

[2] Hinsie, L. E., and Shatzky, J., Psychiatric Dictionary; London, New York, Toronto, Oxford University Press, 1940.

[3] White, W. A., Outlines of Psychiatry, Washington, Nervous and Dental Disease Publishing Co., 1935.

[4] Gellhorn, E., Autonomic Regulations, New York, Interscience Publishers, Inc., 1943.

[5] Henry, G. W., *Am. J. Psychiat.*, 1928, **7**, 135.

† Dibenamine supplied through the courtesy of Dr. W. Gump, Givaudan-Delawanna, Inc., Delawanna, N. J.

[6] Nickerson, M., and Goodman, L. S., *J. Pharm. and Exp. Therap.*, 1947, **89**, 167.

Evaluation of the subjects at the time of administration of the drugs included determinations of blood pressure, pulse rate, skin temperature, sweat secretion, electrical skin resistance, and neurologic and psychiatric status.

Arterial blood pressure was measured in one arm by the standard auscultatory method. Radial pulse rate was counted for 15 seconds.

Skin temperature was measured using a Hardy Dermal Radiometer. Readings were taken from the forehead and from the palm and dorsum of the hand contralateral to the arm in which the infusion was given. Room temperature was also recorded.

Permanent records of palmar sweat secretion were made according to the technic described by Silverman and Powell.[7] Diluted tincture of ferric chloride was painted on the dried, free palm and allowed to evaporate. A print was obtained by holding the palm for 3 min. against a sheet of paper impregnated with tannic acid. Thus the secretory activity of each sweat gland was recorded by a dot of ferric tannate. The number, size and distribution of these dots could be noted readily, and the amount of sweating could be rated according to previously described standards.[8] A faint response (0) included prints varying from almost entirely blank to those showing a light grey shade with fine pinpoint dots. A moderate response (1+) showed thicker dots, darker in shade, arranged in a linear pattern. A strong response (2+) showed black speckles of varying sizes. An intense response (3+) showed blackening with blotches.

Electrical skin resistance was measured using a dermohmeter of the Jasper type.[9] The electrodes were pure silver discs, 1 cm in diameter. No electrolyte paste or jelly was used at either electrode. The resistance was measured between pads of the hallux and the index-finger of the same hand, giving a single reading per patient per examination. This thumb to finger resistance was quickly and simply obtained, and remained constant at an examination. It represents approximately double the value which is obtained if electrolyte paste or jelly is used to reduce the resistance at one electrode.

All neurologic examinations and evaluations of the signs of catatonia were made by the same observer and in a uniform manner. Twelve categories in which abnormalities are frequently found in catatonics had been previously selected. These categories were: posturing, facial immobility, facial grimacing, spontaneous activity, spontaneous speech, activity in response to request, speech in response to questions, strength of manual grip, passive resistance, waxy flexibility, response to pin-prick, and response to supraorbital pressure. In order that comparisons could be made, each subject was rated from 0 to 4+ in each of the 12 categories, according to the amount of deviation from the normal. The results of previous tests were not consulted at the time of subsequent examinations. Consistency of the evaluations was readily demonstrated.

Psychiatric examination consisted of observations and interviews lasting about 30 minutes per patient. All were done by the same examiner, who was already acquainted with the subjects and their histories. Detailed notes were kept.

The drugs employed were sterile sodium amytal, supplied in 0.5 g ampules, and sterile Dibenamine, supplied in ampules containing 500 mg of the drug in 10 cc acidified alcohol-propylene glycol. All drugs were administered intravenously in sterile, isotonic saline solution, supplied in 500 cc flasks, as described below.

*Procedure.* The investigation was carried out in an ordinary 12-bed hospital ward assigned for the purpose. The subjects were kept lightly restrained in bed with their hands exposed for 2 hours preceding the testing, which was begun at about 9:00 a.m. Breakfast and lunch were withheld. After pre-injection observation (blood pressure, pulse rate, skin temperature, palmar sweat secretion, electrical skin resistance, neurologic and psychiatric status), intravenous infusions were started in all subjects, with 400 cc saline in the flasks. When the infusions were running well, 0.5 g of sodium amytal was added to the saline in each of 3 flasks, and mixed well. Similarly, Dibenamine in dosage of 5 mg per kg of body weight of the subject was added to the saline in each of 3 other flasks, and mixed well. The remaining 3 subjects received only saline. The infusion rate was adjusted to approximately 100 drops per minute, so that 1 to 1½ hours were taken for administration of the entire volume. In 4 subjects, whose reactions were inadequate after 350 cc of the dilute sodium amytal solution had been given, additional sodium amytal (up to 0.5 g) was slowly injected directly into the infusion tubing. At the end of each Dibenamine infusion, the tubing and vein were flushed by the addition of about 75 cc of saline to the flask. Immediately following the infusions the pre-injection tests and examinations were repeated. The subjects were kept in bed, but beginning that evening regular meals were served. After breakfast the next morning the tests and examinations were given for the third time, and those subjects who had received saline or amytal were allowed up ad lib. Those who had received Dibenamine were kept in bed until the orthostatic hypotension and tachycardia were gone (24 to 72 hours).

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[7] Silverman, J. J., and Powell, V. E., *Am. J. M. Sc.*, 1944, **208**, 297.

[8] Silverman, J. J., and Powell, V. E., *Psychosomatic Med.*, 1944, **6**, 244.

[9] Jasper, H., *J. Neurosurg.*, 1945, **2**, 257.

The first series of infusions was given March 9, 1948. Two weeks later the entire procedure was repeated, but the subjects were rotated with respect to the drug received. Two weeks after the second series of infusions, the third series was given, with the subjects again rotated. Thus, each patient received 3 infusions—one saline, one saline and sodium amytal, and one saline and Dibenamine (5 mg/kg body weight). (The only exception to this protocol was Subject 6, who was not given his third infusion, saline, because his severe feeding problem made it necessary to institute electric shock therapy.) The fourth and final series of infusions was given 2 weeks after the third. Three subjects who had previously responded comparatively poorly to Dibenamine were selected. This time they received Dibenamine in dosage of 7.5 mg per kg body weight (maximum dose was 500 mg), with the remainder of the procedure for them carried out as on the previous occasions.

*Results.* (A) *Blood Pressure* (*B.P.*): Pre-injection resting B.P. ranged from 124/80 to 96/68, with a mean of 115 ± 1.4/76 ± 1.2.[‡] Neither saline nor sodium amytal resulted in significant B.P. changes, the post-injections means having been 109 ± 2.9/72 ± 1.5 and 110 ± 3.6/71 ± 3.1 respectively. The mean B.P. following the 12 Dibenamine infusions was 121 ± 3.3/67 ± 1.8. Although 4 of the Dibenamine infusions were followed by rises in systolic pressure averaging 15 mm each, the increase in the mean is not statistically significant. The mean change in the diastolic pressure was a significant decrease of 8.5 ± 1.0, representing the effect of blockade of sympathetic vasoconstrictor tone. Eighteen hours after Dibenamine administration the mean B.P. was 121 ± 2.7/68 ± 2.2, and cardiovascular effects of the drug were still in evidence. No testing for orthostatic hypotension was done immediately after the Dibenamine infusions. However, the following morning this phenomenon was noted in 3 of the 9 subjects who had received 5 mg/kg and in all 3 who had received 7.5 mg/kg. Orthostatic hypotension had disappeared from all subjects by the third post-injection day.

(B) *Pulse Rate* (*P.R.*): Pre-injection resting P.R. ranged from 60 to 96, with a mean of 73 ± 2.9/min. The saline and the sodium amytal infusions produced no significant alterations in the P.R. Following the 12 Dibenamine infusions the mean resting P.R. was 110 ± 6.1, a mean increase of 37 ± 6.8 over the pre-injection levels. This

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[‡] All means are given ± the standard error of the mean.

TABLE I.
Effect of Dibenamine on Blood Pressure and Pulse Rate in Catatonic and in Normotensive, Non-Psychotic Subjects.

| | Catatonics | Non-psychotic normotensives[10] |
|---|---|---|
| Resting B.P. | 115 ± 1.4 | 124 ± 2.2 |
| | 76 ± 1.2 | 73 ± 1.7 |
| B.P. after Dibenamine | 121 ± 3.3 | 122 ± 3.0 |
| | 67 ± 1.8 | 69 ± 1.9 |
| Change in B.P. | +6 ± 3.1 | —2 ± 2.6 |
| | —8.5 ± 1.0 | —4 ± 1.5 |
| Resting P.R. | 73 ± 2.9 | 78 ± 2.0 |
| P.R. after Dibenamine | 110 ± 6.1 | 84 ± 2.8 |
| Change in P.R. | +37 ± 8.8 | +6 ± 1.6 |

tachycardia lasted 18 to 36 hours. The morning after the infusions the mean P.R. was 96 ± 6.8, with 2 of the 9 subjects who had received 5 mg/kg and 2 of the 3 who had received 7.5 mg/kg still having resting rates over 100. All subjects had orthostatic tachycardia when tested 18 hours after the Dibenamine infusions. This lasted 24 to 72 hours.

It is of interest to compare the B.P. and P.R. effects of Dibenamine in this group of catatonic subjects with previously reported results in non-psychotic, normotensive general hospital patients.[10] (Table I.) The methods and circumstances of both studies were similar. The initial systolic pressure is significantly lower in the catatonics (difference in means = 9 ± 2.6), although there is no significant difference in diastolic pressures. B.P. studies in a larger series showed significantly lower systolic and diastolic pressures in schizophrenic patients compared with students.[11] Following Dibenamine the mean B.P. of the catatonic group is almost identical with that of the non-psychotics. However, although the initial P.R.s in the two groups did not differ significantly, the catatonics showed a very much faster P.R. following Dibenamine.

The response to Dibenamine of catatonic subjects differed from that of non-psychotic normotensives by showing a slight increase in systolic pressure and a large increase in P.R. These differences are suggestive of 2 effects of injected epinephrine which are not blocked by Dibenamine, namely the rise in cardiac output[12] and the tachycardia.[6,12,13] The presence or production of increased epinephrine in the catatonics might explain the observed differences. Further speculation will be deferred until additional studies are completed.

(C) *Skin Temperature.* Forehead, palm, hand dorsum and room temperatures were recorded. Room temperature varied between 20.0° and 22.4°. Although even this small amount of variation was undesirable, it did not appear to affect the skin temperature results significantly. Furthermore, since all three drugs were being administered to the groups of subjects at the same time, comparison of the mean changes of the groups is valid.

The mean pre-injection forehead temperature was 34.1 ± .2°, dorsum temperature was 27.4 ± .5°, and palm temperature was 27.4 ± .6°. No drug produced significant changes in forehead temperatures. Following saline infusions the hands were slightly cooler in all subjects. The mean change was –1.7 ± .5° for the dorsum and –2.5 ± .8° for the palm. This cooling probably represents the effect of the additional 1 to 1½ hours of exposure with relative immobility. After sodium amytal the mean dorsum temperature change was +3.2 ± 1.5° and the mean palm change was +3.0 ± 1.4°. These mean temperatures (increases) differ from those following saline (decreases) by 4.9 ± 1.6° and 5.5 ± 1.6°, both significant differences. This warming of the hands after sodium amytal was noted in 5 of the 6 subjects who were neurologicly improved by the drug, and in 1 of the 3 who were not. Following Dibenamine the mean change in dorsum temperature was + 2.9 ± 1.3° and the mean change in palm temperature was + 3.6 ± 1.2°. These differ from the mean changes after saline by 4.6 ± 1.4° and 6.1 ± 1.4°, both significant differences. The increases in hand temperature after Dibenamine ranged up to 10.3°, and the greater changes were noted in the subjects who were more improved neurologicly by the drug. Eighteen hours after Dibenamine infusions the increase in hand temperature was still present, the mean changes for the dorsum and palm being +4.6 ± .7° and + 5.4 ± .8° respectively. Eighteen hours after the saline and the sodium amytal infusions the hand temperatures were not significantly different from pre-injection values.

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[10] Haimovici, H., and Medinets, H. E., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 163.

[11] Freeman, H., Hoskins, R. G., and Sleeper, F. H., *Arch. Neurol. and Psychiat.*, 1932, **27**, 333.

[12] Hecht, H. H., and Anderson, R. B., *Am. J. Med.*, 1947, **3**, 3.

[13] (a) Acheson, G. H., Farah, A., and French, G. N., *Fed. Proc.*, 1947, **6**, 305. (b) Youmans, W. B., and Rankin, V. M., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 241.

(*D*) *Sweat Function*: Twenty-seven pre-injection palmar sweat prints were taken from 8 subjects. (No prints were obtained from Subject 6 because his pre-injection posturing included tightly clenched fists.) A faint response was found in 15 (55%) pre-injection prints, a moderate response in 6 (22%), a strong response in 2 (8%), and an intense response in 4 (15%). Individual variation between the days of examination was noted. However, 4 subjects usually produced almost entirely blank prints, whereas 2 others had hyperhidrosis consistently and 1 other had hyperhidrosis occasionally. Neither saline nor sodium amytal infusions produced significant changes in the sweating responses, but immediately following the Dibenamine infusions there were no longer any strong (2+) or intense (3+) responses. Of the 11 prints obtained after Dibenamine, 10 were faint (0) responses and 1 was a moderate (1+) response. This inhibition of excessive palmar sweating gradually disappeared. Eighteen hours after injection, Subject 3 again had an intense (3+) response and Subject 9 had a moderate (1+) response.

Palmar sweat prints obtained by the same technic and rated according to the same standards as the prints in this project were reported to have shown faint or moderate responses in 78% of a group of hospital personnel and strong or intense responses in 84% of the neuropsychiatric (mostly psychoneurotic) patients.

(*E*) *Electrical Skin Resistance* (*E.S.R.*): The mean pre-injection E.S.R. for all infusions in the 9 subjects was 192 ± 18 K.[§] Three subjects, the same ones who had excessive sweating, showed E.S.R. values clearly abnormally low for the testing conditions. The mean pre-injection E.S.R. for these 3 was 105 ± 21 K, whereas for the 6 other subjects the mean was 238 ± 17 K. Five subjects showed pre-injection E.S.R. values which were constant for the several series of tests, but in the other 4 there were large spontaneous day to day variations. Neither saline nor sodium amytal infusions were followed by significant changes in the E.S.R. Immediately after the Dibenamine, the mean E.S.R. was 258 ± 25 K, an increase of 66 ± 30 K from the pre-injection mean; 18 hours post-injection the mean was 290 ± 17 K, an increase of 98 ± 24 K. The increases in E.S.R. after Dibenamine were noted to occur almost exclusively in those subjects whose initial resistances were low. Thus for 8 infusions in 6 subjects whose pre-injection values were under 200 K, the mean change immediately following Dibenamine was + 151 ± 40 K and 18 hours later it was + 195 ± 40 K.

The palmar sweat responses and the E.S.R. measurements made in this study and summarized above show a very high correlation. (Spearman method of rank: $r = .48$, $t = 4.4$, and $p =$ less than .001.)

The initial observations were made with the subjects at basal conditions. The very low sweat responses with high E.S.Rs. found in 4 subjects in contrast with the hyperhidrosis and very low E.S.Rs. found in 3 others demonstrate the extremes of these functions which catatonic patients may show. Wide variation in the E.S.Rs. of catatonics may be found in the data of other reports,[14] although the average palm-to-palm E.S.R. of a group of patients in catatonic stupor was found to be about the same as those of normals and of other groups of psychotic subjects.[15] A relation between a large amount of "free energy (or anxiety)" and low E.S.R., and between a small amount of free energy and high E.S.R. has been suggested.[16]

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[§] K. = 1000 ohms.

[14] (a) Richter, C. P., *Arch. Neurol. and Psychiat.*, 1928, **19**, 488. (b) Richter, C. P., *Arch. Neurol. and Psychiat.*, 1929, **21**, 363.

One may speculate regarding the mechanism by which Dibenamine inhibited excessive sweating and raised low E.S.Rs. in the subjects who showed these pre-injection abnormalities. The regions tested (palms and finger pads) have sweating responses which are of the emotional type, specific for conditions of mental stress.[17] Dibenamine therefore may have produced the observed changes indirectly, as a result of a central action in eliminating an "anxiety" factor. Against this explanation is the failure of sodium amytal to have produced a comparable autonomic effect. The second possibility is that Dibenamine acted on sympathetic ganglia or directly on the sweat glands. These would imply either that Dibenamine inhibited a cholinergic function,[18] or that the excessive sweating and low E.S.Rs. were exhibitions of adrenergic activity. There is no evidence to suggest that Dibenamine is anti-cholinergic. However, there is some reason to believe that certain sweating may have an adrenergic component.[19] It is also possible that Dibenamine decreased the sweating and increased the low E.S.Rs. by an action upon the hypothalamic sympathetic mechanism. Although there is no present direct evidence for this explanation, it is in accordance with other features of Dibenamine's central activity, which are described below.

(*F*) *Neurologic Signs and Signs of Catatonia.* Except for the physical signs of catatonia described below, no subject showed an abnormal neurologic status. Deep tendon reflexes ranged from hypo-active to hyper-active, with 3 tense subjects having occasional transient clonus. Pupils were equal bilaterally in all subjects, and one showed mydriasis. None of the drugs administered changed the general neurologic status significantly. However, transient clonus was not observed after sodium amytal or Dibenamine, and all subjects had miosis following Dibenamine.

As previously outlined, the signs of catatonia were observed in each subject and the amount of abnormality present in each of 12 categories was rated from 0 to 4+. It was therefore possible to record not only general impressions of improvement in the catatonia but also changes in each of the categories. In addition, the total number of points of abnormality shown by a subject at an examination may be considered his "catatonic rating" at that time, and used to rank the subject with regard to severity of the catatonic manifestations.

Pre-injection examinations revealed catatonic ratings for the individuals ranging from 13 to 40, with a mean of 26.5 ± 1.3. The following summarizes the pre-injection findings.

All subjects appeared to be content to lie lightly restrained in bed. Abnormal posturing was common, and was present to a marked degree in 3 subjects. Blankness of facial expression was the rule, although 2 subjects usually showed meaningless grimacing and 3 others grimaced occasionally. Spontaneous motor activity was rare in any of the group. No subject except No. 5 (on one occasion) evinced spontaneous speech during pre-injection testing. Subjects 4 and 5 were the only ones who would respond, even in monosyllables, when questioned prior to the injections. When requested or commanded to perform simple motor actions (lift arm, close eyes, open mouth), 4 subjects rarely or never complied, although 2 others were usually completely cooperative. No subject demonstrated more than feeble effort or strength of manual grip when requested, but considerable force was used by 3 subjects to resist certain passive movements. All but 3 subjects occasionally showed variable degrees of waxy flexibility, but none would hold an induced posture for more than several seconds. The responsiveness to pin-prick and to supra-orbital pressure was markedly diminished or absent in 4 subjects, and in 2 others it was increased to a startle reaction.

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[15] Syz, H., and Kinder, E., *Arch. Neurol. and Psychiat.*, 1928, **19**, 1026.

[16] (a) Solomon, A. P., and Fentress, T. L., *J. Nerv. and Ment. Dis.*, 1934, **80**, 163. (b) Darrow, C. W., and Solomon, A. P., *Arch. Neurol. and Psychiat.*, 1934, **32**, 273.

[17] Kuno, Y., *Lancet*, 1930, **1**, 912.

[18] (a) Feldberg, W., and Gaddum, J. H., *J. Physiol.*, 1934, **81**, 305. (b) Dale, H. H., and Feldberg, W., *J. Physiol.*, 1934, **82**, 121.

[19] Haimovici, H., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 40.

Immediately following the saline infusions no subject was significantly changed. The number of points change for each subject ranged from +3 to –3, with a mean of .3 ± .9 points improvement. Eighteen hours later the mean change was .4 ± 1.3 points improvement. Following amytal, 4 of the 9 subjects were improved 11 to 19 points each, and the mean change was 6.6 ± 2.7 points improvement. The most striking result of the amytal infusions was the production of fair to good verbal responsiveness in 4 subjects. There was also considerably less posturing and no grimacing. Spontaneous activity and spontaneous speech remained poor in all except No. 6, who developed marked resistance in addition. Most subjects were generally relaxed by the drug, and no improvement in strength occurred. Responses to noxious stimuli approached normal in 6 subjects, but were less than normal in 3. The improvement which the sodium amytal produced lasted up to 4 hours, except in Subject 6, who was still improved (7 points) the next day. Eighteen hours after sodium amytal infusions, the mean change from the pre-injection status was 1.1 ± 1.1 points improvement.

Following the 12 Dibenamine infusions, the 9 subjects showed improvements ranging from 0 to 19 points each, with the mean change being 9.2 ± 2.5 points improvement. Nine of the 12 infusions resulted in improvements of 8 points or more, and in these subjects the change in status was quite apparent. Posturing persisted in only one subject of the entire group, and grimacing in but one other. Spontaneous activity was clearly increased in 6 instances, with spontaneous speech appearing in 3 to a considerable degree. Five subjects responded well to requests for simple motor activity and 4 had good speech response. Strength of manual grip did not improve significantly. Although Subject 3 developed resistance to passive motions, all 6 subjects showing such resistance prior to drug administration were improved. Waxy flexibility decreased significantly in Subject 1. Three subjects developed more normal responses to pin-prick and to supra-orbital pressure. The improvement following Dibenamine gradually disappeared in most subjects over a period of 24 to 48 hours. Eighteen hours after the infusions, the mean change from the pre-injection status was 5.8 ± 1.7 points improvement. Speech function was the earliest to regress, disappearing a few hours after the infusion. (Subject 4 had a markedly atypical response to Dibenamine. His improvement began 5 minutes after the drug was started, and he remained improved for over three weeks. His pre-test history showed spontaneous remissions.)

Increasing the dosage of Dibenamine from 5 to 7.5 mg/kg of body weight was associated with an increase in the degree of immediate and 18 hour improvement.

Correlations were sought between initial neurologic status and initial abnormal autonomic functions, and between change in neurologic status and changes in autonomic functions. Because the series was small, statistically significant correlation is difficult. However, a significant coefficient of correlation was found between neurologic improvement and increase in hand temperature after Dibenamine. For the 12 infusions, $r = .5$ $t = 1.9$, and $p = .08$; and for 11 infusions (omitting Subject 4, whose response was atypical), $r = .74$, $t = 3.3$, and $p = .01$. Similar coefficients computed on the changes after sodium amytal are very low.

(*G*) *Psychiatric Examinations.* Without going into any details, the group of patients may be described as representative of the negative variety of schizophrenic reactions—catatonic type, moderately severe. There was no psychiatric change following saline, but sodium amytal brought about definite lessening of tensions. With the verbal mobilization that developed in 4 subjects after amytal, many schizophrenic (hebephrenic and paranoid) symptoms previously not elicitable were observed. When the catatonia was dissolved, there was evidence of ego-centered and introspective concern, with little interest in the surroundings. At a later stage 4 subjects were moderately sleepy, and 1 was deeply so. Dibenamine, in contrast, produced definitely increased interest in surroundings, increased spontaneity, improved responsiveness to environmental stimuli, and no sleepiness. As with sodium amytal, every patient who had speech function after Dibenamine gave expression to hebephrenic or paranoid mental content.

The mechanism by which Dibenamine produced these changes in the catatonia may be considered. In non-psychotic individuals central effects have been observed after Dibenamine.[10,12] Drowsiness is common, and mental confusion with restlessness, or irritability, emotional lability, hallucinations, paramnesia or perseverations have been noted in some patients. Convulsions can be produced by rapid administration in animals[6] and were reported to have occurred in one patient.[12] Nickerson and Goodman state that the central stimulant action of Dibenamine is quite unrelated in time and mechanism to the adrenergic blocking activity, and that almost identical central effects can be produced by the hydrolysis product, N,N-dibenzylethanolamine, which has no adrenergic blocking action.[20] Investigation of this and other related compounds should be illuminating. Malononitrile, $CH_2(CN)_2$, has been reported to produce an effect on psychic functions in mental disorders and to stimulate nucleoprotein production in nerve cells.[21] Although flushing of the face resulted from malononitrile administration, there was no observed change in B.P. The cyanide radical can cause circulatory changes and has been shown to produce vasodilation, probably by paralysis of smooth muscle in vessel walls.[22] Catatonia has been observed to dissolve upon I.V. administration of 1/50 N. sodium cyanide solution.[23] While Dibenamine may have a direct effect upon nerve cell metabolism, it is not impossible that the improvement in the catatonia which followed its use also may be associated with alterations in cerebral circulation, particularly in the region of basal arterial supply. The correlation between neurologic change and change in skin temperature suggests at least parallel development of the increase in peripheral circulation and the improvement in the catatonia. The most recent study does not support the contention that the total cerebral blood flow in schizophrenics is abnormal.[24] The effect of Dibenamine on the circulation of blood in the brain of animals is being studied.

In this investigation, the responses to Dibenamine and the responses to sodium amytal were different. Only one of the 4 subjects who were greatly benefited by sodium amytal was among the 4 who were most improved by Dibenamine. Furthermore, the types of responses produced by the two drugs were characteristically dissimilar, the amytal changes being especially verbal and responsive and the Dibenamine changes being increased spontaneity and interest in surroundings. It would not appear that the mechanisms of action of these drugs are the same.

(*H*) *Toxic and Side Effects.* Few of the previously reported toxic effects of Dibenamine[10,12] were observed in these subjects. There were no instances of phlebothrombosis, although Subject 4 complained of pain along the vein throughout the infusion. Only one subject appeared to be nauseated or vomited (No. 3, who had brief retching after each of the 2 Dibenamine infusions given him. He was an intermittent soiler, and also showed loss of sphincter control following one administration of Dibenamine). Increased drowsiness was not produced. When Subject 2 exhibited masturbatory activity after Dibenamine (4th series of infusions), he and the 2 other patients who had received the drug at the same time were found to have priapism. The subjects had not been examined for this in the previous tests.

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*Comments*: There is psychiatric dissatisfaction with schizophrenia as a nosologic entity and particularly with the sub-classifications as they now stand. The stigmata characteristic of catatonia are physiologic and can be experimentally produced or abolished. When sodium amytal or Dibenamine dissolves the catatonia in patients with schizophrenic reactions, catatonic type, hebephrenia or paranoia appears. Catatonia, which is an abnormal physiologic state, is therefore actually at a different descriptive level from either hebephrenia or paranoia, which are psychopathic states. Further discussion of this matter is reserved for later publication.

There is considerable experimental and clinico-pathological evidence suggesting that the manifestations of catatonia are mediated by the hypothalamus[25] and/or the basal ganglia.[26] We will not review this material in the present paper. However, it can be pointed out that both motor and autonomic centers are located in this region of basal arterial supply, and that motor and autonomic disturbances frequently accompany clinical and experimental lesions in this area. Catatonia and epidemic encephalitis supply many examples of co-existence of such motor and autonomic abnormalities. The physiologic basis for the association of these abnormalities may be the common blood supply to the hypothalamus and the basal ganglia.

*Summary*: 1. Certain autonomic functions (B.P., P.R., skin temperature, palmar sweat response, electrical skin resistance), the signs of catatonia, and the psychiatric status of 9 patients with schizophrenic reactions, catatonic type, were evaluated before and after saline, sodium amytal, and Dibenamine infusions.

2. Pre-injection autonomic abnormalities were low systolic B.P., cool extremities, and either very low sweat responses with high E.S.Rs., or hyperhidrosis with very low E.S.Rs.

3. Saline produced no significant changes.

4. Sodium amytal infusions were followed by increased hand temperatures and improvement in the catatonia, especially in the verbal spheres. The subjects became relaxed, showed little interest in surroundings. In those 4 who developed sufficient speech function, hebephrenic or paranoid ideas were expressed. All changes were transient.

5. Dibenamine infusions were followed by a slight increase in systolic B.P. and a large increase in P.R., neither of which changes have been reported in non-psychotic subjects after Dibenamine. Excessive sweating was inhibited, and low E.S.Rs. were raised. The hands became warmer. Significant improvement in the signs of catatonia was noted in most subjects, especially (and in contrast to the effects of amytal) production of increased spontaneous activity and increased interest in surroundings. There was correlation between warming of the hands and the neurologic improvement. As with amytal, verbalization revealed hebephrenic and paranoid ideas. Toxic effects were infrequent and minor. All effects were transient, but of longer duration than with amytal.

6. The possible mechanisms by which Dibenamine inhibits sweating and by which it improves catatonia are discussed.

7. A difference in the descriptive level of catatonia from that of hebephrenia and paranoia is pointed out.

8. The common blood supply of the hypothalamus and the basal ganglia is suggested as a possible basis for the clinical association of certain motor and autonomic abnormalities.

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### 16680

# Suppression of Local Tissue Reactivity (Shwartzman Phenomenon) by Nitrogen Mustard, Benzol, and X-Ray Irradiation.

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When a filtrate of *Eberthella typhosa* is injected intradermally into a rabbit and 24 hours later injected intravenously, there develops a severe hemorrhagic necrotic reaction at the site of the intradermal injection within a period of 2-6 hours. Shwartzman[1] made his original observations on this reaction in 1927. It was soon found[2] that many unrelated bacteria were capable of producing the reaction, including the *Neisseria intracellularis* (Meningococcus), as well as non-bacterial substances, such as agar and starch. Although a certain small number of rabbits are naturally resistant to the reaction, a filtrate or endotoxin of a virulent meningococcus will consistently produce strong reactions in 90-100% of rabbits tested.

Although the histological reaction is basically one of vascular injury, opinions[2,3] differ as to the nature of the Shwartzman phenomenon. There is agreement, however, that it is a non-specific reaction which does not involve the known immunological mechanisms (agglutinins, precipitins, etc.). Because of the known depressive effect of Benzol, X-ray irradiation, and nitrogen mustards on the parenchymatous elements of the blood-forming organs[4-7] and on the reticuloendothelial system,[8-15] these agents were employed in an effort to throw light on the nature of the Shwartzman reaction.

*Methods and Materials.* The meningococcus filtrate and meningococcus endotoxin which were used throughout this study were prepared as follows:†

*Meningococcus filtrate*: Avirulent Type I strain of meningococcus was grown in 16 oz. flat-sided bottles for 24-30 hours on casein digest agar[16] enriched with cystein. The growth of each bottle was washed off with 10 cc of physiological saline in 0.4% phenol and centrifuged at approximately 3000 RPM for 20 minutes. The clear supernatant was passed twice through Berkefeld candle filters, adjusted to pH 7.8 and cultured for sterility.

Meningococcus endotoxin: The remaining sediment was resuspended in water (20 cc per culture bottle), adjusted to pH 8.2, incubated for 2 hours, placed in the icebox overnight, readjusted to pH 7.8, placed in a water bath at 60°C for 1 hour and 45 minutes, and cultured for sterility.

Fresh batches of the filtrate and the endotoxin were prepared approximately every 3 weeks and routine weekly cultures of both were sterile. The endotoxin was slightly more potent intravenously but both were found equally effective in eliciting severe hemorrhagic reactions in over 90% of the rabbits used. The control animals tested in any given group of rabbits received the same dose of the same substance by the same route as the treated animals of that group. The majority of the animals received the filtrate as the intradermal injection (*the skin preparatory factor*) and the endotoxin intravenously (*the intravenous reacting factor*).

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*Animals*: Male albino rabbits weighing 2-3 kg were used. The hair on the abdomen was removed with electric clippers. No depilatories were used; no shaving was done.

*Intradermal injections*: Intradermal injections of 0.3 cc of the undiluted meningococcus filtrate or endotoxin were made in 3 different areas, the epigastrium, the right and left lower quadrants. The primary reactions resulting from the intradermal injections usually consisted of erythema and varying degrees of edema which would fade gradually over a 48-hour period if not followed in 24-48 hours by injection of the intravenous reacting factor.

*Intravenous injections*: From 20-26 hours after the intradermal injection, 2 cc of the undiluted meningococcus endotoxin per rabbit or 2 cc of the undiluted meningococcus filtrate per kg body weight was injected intravenously through the ear vein. A small percentage of the rabbits died within the first few hours after this injection from the inherent toxicity of the undiluted filtrate or endotoxin. Dilution was avoided to insure as high a percentage of severe reactions as possible.

*Results*: The effects of various agents were studied to determine their suppressive action on the Shwartzman phenomenon.

*Results in Untreated Control Animals.* Twenty-one normal untreated rabbits were used as controls. Of these, 19 developed severe hemorrhagic necrotic reactions measuring 3 - 6 cm in diameter at all 3 sites of the intradermal injections. One rabbit of the 21 was resistant and developed no reaction and the other rabbit died at the end of 4 hours post intravenous and had no skin reaction at the time of death. There was no consistent correlation noted between the intensity of the primary reaction from the intradermal injection itself and the severity of the hemorrhagic reaction after the injection of the intravenous reacting factor.

*Results in Treated Animals.* (1) The following reagents employed in near-lethal doses exerted no suppressive action on the Shwartzman reaction: benadryl, (10 mg IV *q* 3 h), urethane, (1 Gm bid subcut, for 1-14 days), penicillin G, (500,000 u and 1,000,000 u IM daily x 4 days), crude penicillin, (35 cc bid x 2 days), streptomycin, (0.5 Gm and 1.0 Gm daily IM x 4 days), British anti-lewisite, (50-100 mg bid subcut), sodium ascorbate, (75 mg daily subcut x 10 days), alpha tocopherol, (25 mg subcut daily x 10 days), protamine, (20 mg bid IV x 2 days), toluidine blue, (20 mg bid IV x 2 days), and mapharsen (13 mg/kg IV 1 inj.). Total thyroidectomy 3 weeks before the reaction and bleeding (30 cc daily x 2 days) also had no suppressive effect.

(2) *Following Total Body X-ray Irradiation.* When 13 rabbits were exposed once to 800 r total body irradiation 3-7 days before injection of the intravenous reacting factor there was almost complete suppression of the reaction in 7 of the rabbits and complete suppression of the reaction in the other 6 (Table I). One rabbit received a total body dose of only 300 r, 3 days before, and showed no suppression of the reaction. Two rabbits received 800 r total body irradiation apiece immediately before the injection of the intravenous reacting factor and these likewise showed no suppression of the reaction.

(3) *Following Benzol.* Benzol mixed with equal parts of sterile olive oil, was injected subcutaneously in doses of 1.0-2.0 cc of benzol per kg body weight (2.0-4.0 cc per kg of the benzol-olive oil solution) once daily for 7-13 days before injection of the intravenous reacting factor. The benzol was given daily until the rabbits showed outward signs of toxicity such as weight loss, refusal of food and lethargy. As noted in Table I, complete

TABLE I.
Effects of Benzol and X-ray Irradiation on the Shwartzman Phenomenon.

| Dosage schedule | | | Reactions following inj. I.V. reacting factor (4-6 hr) | | | |
|---|---|---|---|---|---|---|
| Amt. | Days before I.V. reacting factor* | No. of rabbits | None | ++ | ++++ | Deaths |
| Benzol—1.0-1.5 cc | 4 | 1 | | | | 1 |
| per kg daily | 7 | 4 | 2 | | | 2 |
| Subcut | 8 | 3 | 3 | | | |
| | 9 | 1 | 1 | | | |
| | 13 | 1 | 1 | | | |
| 2.0 cc per kg | 7 | 1 | | | | 1 |
| | 7 | 1 | 1 | | | |
| Totals | | 12 | 8 | 0 | 0 | 4 |
| X-ray total body irradiation | | | | | | |
| 800r | 0 | 2 | | | 2 | |
| 300r | 3 | 1 | | | 1 | |
| 800r | 3 | 2 | | 2 | | |
| 800r | 4 | 10 | 6 | 4 | | |
| 800r | 7 | 1 | | 1 | | |
| Totals | | 16 | 6 | 7 | 3 | |

None = no reaction.
++ = suppressed hemorrhagic reaction (1 cm).
++++ = severe hemorrhagic reaction (3-6 cm).
* = Meningococcus filtrate or endotoxin.

TABLE II.
Effect of Single Injection of Methyl-bis Nitrogen Mustard Intravenously on the Shwartzman Phenomenon.

| Dosage schedule | | | Reactions following inj. I.V. reacting factor (4-6 hr) | | | |
|---|---|---|---|---|---|---|
| Amt. | Days before I.V. reacting factor* | No. of rabbits | None | ++ | ++++ | Deaths |
| $\frac{2\text{ mg}}{\text{kg}}$ | 0 | 2 | | | 2 | |
| | 1 | 2 | | | 2 | |
| | 2 | 2 | 1 | 1 | | |
| | 3 | 9 | 9 | | | |
| | 4 | 4 | 3 | | | 1-1 hr |
| | 8 | 2 | 1 | 1 | | |
| | 11 | 2 | | 1 | 1 | |
| | 12 | 1 | | | 1 | |
| | 15 | 1 | | | 1 | |
| $\frac{1\text{ mg}}{\text{kg}}$ | 3 | 2 | 2 | | | |
| $\frac{0.5\text{ mg}}{\text{kg}}$ | 3 | 4 | | | 3 | 1-4 hr (no reac.) |
| Controls | | 21 | 1 | | 19 | 1-4 hr (no reac.) |

None = no reaction.
++ = suppressed hemorrhagic reaction (1 cm).
++++ = severe hemorrhagic reaction (3-6 cm).
* = Meningococcus filtrate or endotoxin.

suppression of the Shwartzman reaction was obtained in all 8 of the rabbits tested. Four rabbits, of the original 12 started with in this group, died during the period of benzol preparation.

(4) *Following Nitrogen Mustard.* Nitrogen mustard (methyl bis chlorethyl amine hydrochloride) was given in a single injection by the intravenous route with doses ranging from 0.5-2.0 mg/kg body weight, from 0-15 days before injection of the intravenous reacting factor. As can be seen from Table II,

there was no protection afforded when the mustard was given either at the same time as the intravenous reacting factor or when it was given on the day of the intradermal injection 20-26 hours before the intravenous reacting factor. When 2 mg/kg of nitrogen mustard was given 2 days before the intravenous reacting factor, there was partial suppression of the reaction in 1 rabbit and complete suppression in the other. When the nitrogen mustard was given in doses of 1-2 mg/kg 3-4 days before the injection of the intravenous reacting factor, the effects of the nitrogen mustard became more pronounced and there was complete suppression of the reaction in 14 of the 15 rabbits so treated, with the 15th rabbit dying during the first hour after injection of the intravenous reacting factor. When given 8-11 days before, the suppressive effect of the mustard was beginning to wane and in only one of 4 rabbits was there complete suppression, in 2 rabbits there was partial suppression and in 1 rabbit there was no suppression. When given the mustard 12 and 15 days before the injection of the intravenous reacting factor, the rabbits had completely regained their ability to react and, like the control rabbits, had severe hemorrhagic reactions. When a 0.5 mg/kg dose of mustard was used, no suppressive effect was noted.

*Discussion.* It is considered significant that the Shwartzman phenomenon of local tissue reactivity, even though a non-specific reaction, can be completely suppressed by nitrogen mustard, benzol, and X-ray, whose individual effects on blood-forming organs and the reticulo-endothelial system are so nearly identical. It is postulated that the mechanism of suppression by these agents is exerted through their specific but common suppressive action on the reticulo-endothelial system, primarily the vascular endothelium. These endothelial cells being rendered anergic are not able to react to the active principles in a way that otherwise would be self-destructive.

The Shwartzman phenomenon may then be interpreted as a local intracellular defensive but self-destructive reaction of the vascular endothelium to the bacterial active principles and not a reaction resulting from the direct toxic effects of these bacterial products on the cells. Thus, when the ability of the cell to react is interfered with or suppressed in a specific way, as exerted by nitrogen mustard, benzol, or X-ray, the integrity of the cell is maintained and a destructive process avoided.

It is suggested that this study provides an experimental basis for a new therapeutic concept in the treatment of diseases involving tissue and vascular reactivity to known and unknown toxins. This concept would be directed toward suppressing the ability of the vascular endothelium to react adversely to whatever circulating toxin might be the inciting agent. A group of these diseases would include active rheumatic fever, acute, subacute, and chronic disseminated lupus erythematosus, periarteritis nodosa, generalized vascular diseases due to hypersensitivity reactions to drugs, sera or vaccines, and probably dermatomyositis, rheumatoid arthritis, and acute and subacute glomerulonephritis. A recent case report by Osborne and associates[17] of the successful response of a case of chronic disseminated lupus erythematosus to nitrogen mustard would seem to provide confirmatory evidence in support of the therapeutic concept postulated above.

*Summary.* 1. The Shwartzman phenomenon was studied in rabbits using a meningococcus filtrate and meningococcus endotoxin. 2. The following agents were without suppressive effect on the Shwartzman phenomenon: benadryl, urethane, crude penicillin extract, penicillin G, streptomycin, mapharsen, BAL, vitamin C, alpha tocopherol, thyroidectomy, and partial exsanguination. 3. The reaction was completely suppressed by pretreatment of the rabbits with nitrogen mustard or benzol. It was also completely suppressed in some rabbits and partially in others by pretreatment with total body X-ray irradiation. 4. It is suggested that this study provides an experimental basis for a new therapeutic concept in the treatment of diseases involving tissue and vascular reactivity.

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### 16681

# Conglutinating Complement Absorption Test Compared with Hemolytic Complement-Fixation Reactions Using Q Fever Immune Bovine Serum.

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The difficulty of evaluating serodiagnostic tests performed on animal serums of various species is well known. In complement-fixation tests sensitivity is largely sacrificed for specificity, and modifications, such as alterations of the units of complement, varying the time and temperature of the incubation period, have not proved entirely satisfactory.

Hole and Coombs[1] reinvestigated the phenomenon of conglutination, first described by Bordet and Streng,[2] and developed a conglutinating complement absorption test[3] which they applied to a study of glanders infection in horses.[4] This test is based on the observation that when heat-inactivated bovine serum and fresh (unheated) horse, cat or pig serum are added to a suspension of sheep red cells, the cells clump together, or conglutinate. Bovine serum plays a dual role by furnishing a natural antibody to the sheep cells for their sensitization, and also a substance termed conglutinin. The reaction of the antibody and sheep cells has the property of absorbing complement. Obviously, the complement in this case must be of a nonhemolytic nature, otherwise the sheep cells would be lysed rather than conglutinated. Fresh horse, cat or pig serum provides such a complement. Thus, conglutinin acts on the sensitized cells which have also absorbed complement and causes them to conglutinate. It is necessary that all 4 of the above components be present before conglutination occurs. In the test proper a known antigen and a suspected immune serum are mixed in the presence of a conglutinating complement. Later the conglutinating system (inactivated bovine serum and sheep red cells) is added to detect the presence of unabsorbed complement. In this test, conglutination may be considered analogous to hemolysis in the hemolytic complement-fixation test.

In the report which follows, the test was applied to Q fever infections in cattle and the results were compared with those obtained by two widely used technics of the hemolytic complement-fixation reaction.

*Technics of the Tests. Conglutinating Complement Absorption Test (C.C.A.T.) Diluent.* Physiological salt solution adjusted to pH 7.3 with 0.005 *M* phosphate buffer was used throughout the study.

*Sheep red cell suspension.* Defibrinated sheep's blood was stored in modified Alsever's solution.[5] Before use the cell suspension was standardized spectrophotometrically and diluted to 0.25%.

*Conglutinin.* As mentioned previously, bovine serum served as the source both of conglutinin and antibody for sheep red cells. Routinely bovine serum is titrated for conglutinin only. Since the sheep cell antibody is always much higher in titer, a sufficient amount of antibody to sensitize the cells is present in the quantity of serum required to cause conglutination.

Conglutinin is not destroyed at ordinary inactivation temperatures. It is easily preserved by sealing small volumes in glass ampoules and, without adding chemical preservatives, storing in the $CO_2$ icebox. For titration, the serum is thawed, inactivated for 30 minutes at 56°C and diluted 1:30 with the diluent. Graduated amounts are placed in tubes, brought to total volumes of 1.2 ml with diluent, and complement (0.4 ml of a 1:40 dilution), and cells (0.4 ml) are then added. Following an incubation period of 30 minutes at 37°C, the tubes are centrifuged for a few minutes at a low speed. After shaking the tubes, the conglutinated cells appear as small clumps in a clear supernate. The absence of conglutination is evident from the homogeneous suspension of cells. The smallest quantity of conglutinin giving complete conglutination is taken as one unit. Four units, contained in 0.4 ml, are employed in the test.

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*Conglutinating complement.* Fresh horse serum, preserved by storing small amounts in sealed glass ampoules in the $CO_2$ icebox, was used. Its activity was satisfactory even after a period of several weeks. For titration, increasing amounts of complement (in increments of 0.05 ml) of an appropriate dilution are added to 0.4 ml antigen, with sufficient diluent to bring the total volumes to 1.2 ml. After incubation for 30 minutes at room temperature, 0.8 ml of a mixture of equal parts 0.25% sheep cells and bovine serum (adjusted to contain 4 conglutinating units) is added to each tube. Following a 30 minute incubation period at 37°C the tubes are centrifuged, shaken and read. The smallest amount of complement giving complete conglutination is taken as one unit. Two units, contained in 0.4 ml, are employed in the tests.

*Antigens.* The Q fever antigen, consisting of washed, formalinized suspension of the American Nine Mile strain of *Coxiella burnetii,* was prepared according to the method described by Plotz[6] but with certain modifications, the details of which have been presented elsewhere.[7] The antigens of *Rickettsia typhi* (murine typhus) and *Rickettsia akari* (rickettsialpox) were similarly prepared and used to control specificity. These two were used because at the time they were the only washed rickettsial body antigens available. The 3 antigens were originally titrated with their respective immune guinea pig serums. The Q fever antigen was also titrated with a positive bovine serum which gave titers comparable to those obtained with the guinea pig serum.

The "optimal" antigen dose must be used with the C.C.A.T. because of the tendency for prozone reactions. This dose was determined by cross titration of various dilutions of the antigen in the presence of various dilutions of the immune serum. The smallest amount of antigen showing maximal conglutination titers, without demonstrating prozones, is the optimal dose.

*Serums.* Immune serums from 34 Q fever infected cattle were obtained through the courtesy of Dr. R. J. Huebner of the National Institute of Health, Bethesda, Md.

*Technic of the C.C.A.T.* Twofold dilutions of the inactivated serum to be tested are prepared. Two units of complement and the optimal antigen dose are added and the tubes incubated at room temperature for 30 minutes. After the addition of the conglutinating system the tubes are incubated at 37°C for an additional 30 minutes. The tubes are then centrifuged and read. Appropriate controls for the test serum, antigen and complement are always included.

In the original technic of Hole and Coombs[3] 0.4 ml of each test component was used, thus making a total volume of 2.0 ml per tube. In the present study one-half these volumes were used and no interference with the sensitivity, specificity or the reading of the results was observed. Also, preliminary trials indicated that the results were equally satisfactory when stored cells and buffered diluent were used, instead of the fresh cells and unbuffered physiological salt solution suggested in the original method.

*Hemolytic Complement-Fixation Methods.* Two methods were used, the so-called warm-bath method, suggested by Huebner,[8] and the icebox method.

*Warm-bath method.* Test components: serum, antigen, complement, amboceptor and fresh sheep red cells in 0.2 ml quantities each.

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TABLE I.
Results of C.C.A.T. and Complement-fixation Titrations of Positive Q Fever Cattle Serums with Q Fever, Murine Typhus and Rickettsialpox Antigens.

| No. of serums tested | C.C.A.T. titers* | | | | Complement-fixation titers* | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Warm-bath method | | Ice-box method | |
| | Q fever | Murine typhus | Rickett-sialpox | Control† | Q fever | Control | Q fever | Control |
| 1 | 1:16 | — | — | — | — | — | 1:16 | — |
| 1 | 1:64 | — | — | 1:8 | — | — | 1:8 | — |
| 2 | 1:64 | — | — | — | — | — | 1:8<br>1:16 | — |
| 1 | 1:128 | — | — | 1:32 | — | — | 1:16 | — |
| 1 | 1:128 | 1:16 | | 1:64 | — | — | 1:32 | 1:8 |
| 1 | 1:128 | — | — | — | 1:16 | — | 1:64 | — |
| 2 | 1:128 | — | | — | 1:8 | — | 1:32 | — |
| 1 | 1:256 | — | | 1:8 | 1:16 | — | 1:32 | — |
| 3 | 1:256 | — | | — | 1:16 | — | 1:32<br>1:64(2) | — |
| 1 | 1:512 | 1:8 | | 1:128 | — | — | 1:64 | — |
| 2 | 1:512 | — | | — | 1:4<br>1:8 | — | 1:64 | — |
| 1 | 1:512 | 1:16 | | 1:32 | 1:8 | — | 1:128 | — |
| 1 | 1:512 | — | | — | 1:8 | — | 1:128 | — |
| 4 | 1:512 | — | — | — | 1:16 | — | 1:64(3)<br>1:128 | — |
| 5 | 1:512 | — | | — | 1:32 | — | 1:64(2)<br>1:128(3) | — |
| 1 | 1:1024 | — | | — | 1:16 | — | 1:256 | — |
| 2 | 1:1024 | — | | — | 1:32 | — | 1:128<br>1:256 | — |
| 3 | 1:1024 | — | | — | 1:64 | — | 1:128<br>1:256(2) | — |
| 1 | 1:2048 | — | | 1:16 | 1:32 | — | 1:256 | — |

* The highest dilution of the serum in which complete, or near-complete (3+ or 4+) fixation was observed is shown.

† The dose of complement used is not always the same for each antigen. Serum controls were always tested with the highest dilution of complement used. It is reasonable to assume that if a serum is not anticomplementary in the presence of a small amount of complement it would not be anticomplementary with larger quantities of complement.

Unbuffered physiological salt solution, and 2% suspension of red cells are used. Varying amounts of amboceptor are titrated in the presence of a 1:30 dilution of complement for one hour at 37°C. Complement is titrated in the presence of 2 units of antigen after fixation for the same period of time and temperature; 2 units of amboceptor and cells are added and a period of one hour at 37°C is allowed for hemolysis. Two "full units" of complement contained in 0.2 ml are used in the test.

The test consists of mixing twofold serial dilutions of the suspected immune serum with 2 units of antigen and 2 "full units" of complement. Fixation is allowed to take place for one hour at 37°C. The sensitized cells are then added and the tubes reincubated for one hour at 37°C. All tubes are stored overnight at 4 to 6°C and readings are made the following morning.

*Icebox method.* This method is similar to that described above except that 2 "exact units" of complement are used and a period of 24 hours at 4 to 6°C is allowed for fixation. The tubes are read after the addition of the sensitized cells, followed by incubation of one hour at 37°C.

In both complement-fixation tests the antigens were titrated with their respective immune guinea pig serums.

On each serum specimen all 3 titrations, the C.C.A.T. and the 2 hemolytic complement-fixation tests, were performed on the same day with the identical dilutions. The C.C.A.T., using Q fever, murine typhus and rickettsial-

TABLE II.
Results of C.C.A.T. and Complement-fixation Titrations of Positive Q Fever Human and Guinea Pig Serums with Q Fever, Murine Typhus and Rickettsialpox Antigens.

| | | | | | | Complement-fixation titers | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | C.C.A.T. titers | | | | Warm-bath method | | Ice-box method | |
| Serum | Lowest serum dilution tested | Q fever | Murine typhus | Rickett-sialpox | Control | Q fever | Control | Q fever | Control |
| Human 1 | | 1:1024 | 1:128 | 1:128 | 1:128 | 1:8 | 1:8 | 1:64 | 1:32 |
| ,, 2 | | 1:256* | — | — | 1:8 | 1:64 | — | 1:128 | — |
| ,, 3 | | 1:512 | — | — | — | 1:64 | — | 1:128 | — |
| ,, 4 | | 1:512* | — | — | 1:16 | 1:32 | — | 1:64 | — |
| ,, 5 | 1:8 | 1:1024* | — | — | — | 1:256 | — | 1:512 | — |
| ,, 6 | | 1:256 | — | — | — | 1:32 | — | 1:128 | — |
| Guinea pig No. 3 Nine-Mile | | 1:2048 | — | — | 1:8 | 1:512 | — | 1:1024 | — |
| Guinea pig No. 39 Nine-Mile | | 1:2048 | — | — | — | 1:512 | — | 1:1024 | — |
| Guinea pig No. 5 Nine-Mile | | 1:2048 | — | — | — | 1:512 | — | 1:1024 | — |
| Guinea pig Henzerling | | 1:2048 | — | — | 1:32 | 1:512 | — | 1:1024 | — |

* Prozone.

TABLE III.
Results of C.C.A.T. and Complement-fixation Titrations of Serums from Cattle Having No Known Contact with Q Fever, with Murine Typhus, Q Fever and Rickettsialpox Antigens.

| | | | | | | Complement-fixation titers | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | C.C.A.T. titers | | | | Warm-bath method | | Ice-box method | |
| Serum | No. serums tested | Q fever | Murine typhus | Rickett-sialpox | Control | Q fever | Control | Q fever | Control |
| Brucella negative | 2 | — | — | | — | — | — | — | — |
| | 6 | — | — | | — | — | — | 1:4 | — |
| | 2 | — | — | | — | — | — | 1:8 | — |
| Brucella positive | 3 | — | — | — | — | — | — | 1:8 | — |
| | 3 | — | — | — | — | — | — | 1:16 | — |
| | 1 | — | — | — | — | — | — | 1:32 | — |

pox antigens, and the 2 types of hemolytic complement-fixation tests, using Q fever antigen only, were performed on human, guinea pig and cattle serums. With the C.C.A.T. pools of normal human serums, human syphilitic serums and normal guinea pig serums (all used in dilutions of 1:4) were negative with the 3 antigens. Murine typhus and rickettsialpox guinea pig serums, tested with their respective antigens, gave titers comparable to those obtained with the icebox method for complement fixation test.

*Discussion of Results.* Considering the results of the Q fever conglutinating complement absorption test from the point of specificity, it is seen from the data shown in Tables I and II that, unless also anticomplementary, no Q fever immune serum (cattle or otherwise) reacted with the murine typhus or rickettsialpox antigens. Of the 17 serums obtained from cattle having a negative history of Q fever (Table III), none were positive when examined by the C.C.A.T.

It will be noted from the data summarized in Table I that with the positive Q fever cattle serums the order of increasing sensitivity is: the warm-bath method, the icebox method, and the C.C.A.T. Serums submitted to the C.C.A.T. show titers from 4 to 8 times higher than those obtained with the icebox complement-fixation method, and these in turn are from 4 to 8 times higher than those given by the warm-bath method. A similar picture is noted in Table II with human and guinea pig Q fever immune serums. Here, however, the differences in titers are of an order of from 2 to 4 times greater rather than 4 to 8. This apparently greater increase in sensitivity when testing cattle serum is, as yet, unexplained.

The question arises whether the use of cattle serum, as source of the immune body, causes irregularities in the results of the C.C.A.T. One would expect that conglutinin and normal antibodies for sheep cells introduced in excess of the standard amounts would tend to lower the antibody titer rather than cause the observed increase. Normal sheep amboceptor probably was present in the serums studied but no attempt was made to adjust the amount of amboceptor employed in the test. It is doubtful whether any conglutinin persisted until the date of testing since storage conditions are unfavorable for that particular component, and bearing in mind its relatively low titer (1:40 or 1:48), dilution of the serums beyond that point undoubtedly removed any effects which may be attributed to the introduction of additional conglutinin into the reaction mixture.

It is noteworthy that the titers of the Q fever, rickettsialpox and murine typhus antigens, as determined by the icebox complement-fixation method, were 1:32, 1:32 and 1:16 respectively, while the "optimal" C.C.A.T. titers on the same antigens were 1:120, 1:4 and 1:4 respectively. The Q fever antigen titer of 1:120 was determined with both bovine and guinea pig immune serums.

From the icebox complement-fixation results obtained with the Q fever negative-Brucella negative cattle serums (Table III) it would seem possible to adopt a lower limit of test significance, *i.e.*, a positive result exhibited in serum dilutions only as high as 1:8 would not be considered significant. But the inadvisability of imposing such an arbitrary limitation is demonstrated by the higher titers offered by the Q fever negative-Brucella positive serums (Table III).

The data presented indicate that the Q fever conglutinating complement absorption test may prove superior, both in sensitivity and in specificity, to either of the hemolytic complement-fixation tests employed to date. It is realized that numerous specimens of serum from cattle suffering from infections other than Q fever and Brucellosis should be examined before placing too much confidence in the results of the C.C.A.T. Such a study is now being undertaken and serums of goats, sheep, swine, dogs and horses will be included.

*Conclusions.* Preliminary studies in testing Q fever immune bovine serums indicate that the conglutinating complement absorption test is both more sensitive and more specific than are either the warm-bath or icebox methods for carrying out hemolytic complement-fixation tests.

### 16682

# Serum Neutralizing Antibodies to the Infecting Strain of Virus in Poliomyelitis Patients.*†

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There has been very little satisfactory data collected regarding the development of specific neutralizing antibodies in human patients during an infection with one of the poliomyelitis viruses. Tests applied to convalescent or acute and convalescent sera, have usually been made with any convenient laboratory-adapted strain of virus. In most instances, workers have failed to demonstrate rising antibody titers, such as those encountered in many other virus infections, and frequently antibody has been found in high titer during the acute phase of the disease. These unusual and unexpected findings have been attributed usually to: (1) suspected changes in the virus strain, which might have occurred during adaptation to a laboratory animal, (2) the possibility that infection was due to a virus antigenically distinct from that used for the test, and (3) the possibility that the substance or substances responsible for neutralization are of a non-specific nature.

To our knowledge, only two instances have been reported in which a virus has been isolated from a patient and the same virus used to test the acute and convalescent phase sera of that patient for antibodies.[1,2] In both instances the test was qualitative and served only to indicate roughly the presence or absence of antibody. The results were interpreted as indicating that in the *acute stage*, antibody was absent. Antibody was definitely present in the later specimen in the one instance,[2] but the results of the test on the convalescent specimen were considered equivocal in the other.[1]

Beginning 4 years ago, serum specimens and stool specimens were collected from a series of paralytic patients from various parts of California. A large series of viruses was isolated and monkey adaptation attempted, but unfortunately, most of the viruses had a low degree of pathogenicity for monkeys and could not be satisfactorily adapted to give clear-cut results in neutralization tests. At the present time, after using over 200 monkeys, tests of a reasonably satisfactory nature have been completed on the sera from 7 patients. Since the results are fairly consistent they are being reported at this time. Tests are now in progress on sera from other patients.

*Methods.* After isolation of a virus from feces, it was passed in monkeys as a 20% cord suspension, or 10-fold concentrate thereof, for not less than 3 passages, or until most of the inoculated monkeys developed paralysis. At this time, except in the earliest experiments, a pool of 20% cord suspension from several monkeys was concentrated in the ultracentrifuge to approximately one-tenth of its original volume and frozen in dry ice in a series of ampoules. Two or 3 monkeys were then inoculated with a mixture of an equal quantity of normal monkey serum and this frozen virus suspension. This mixture had been incubated one hour at 37°C, then overnight at 5°C; the predetermined conditions of the neutralization test to be performed later. If all monkeys became paralyzed, the virus was considered to be adequately adapted for the neutralization test. A number of viruses had to be discarded, after numerous tests showed that paralysis did not develop consistently.

Following the preliminary assay of pathogenicity, the virus was tested with each of 2

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* Aided by a grant from the National Foundation for Infantile Paralysis.

† We wish to acknowledge the assistance of W. N. Mack, R. G. Givens, and C. R. Harms in performing these experiments.

[1] Trask, J. D., and Paul, J. R., *J. Exp. Med.*, 1933, **58**, 531.

[2] Sabin, A. B., *J.A.M.A.*, (June 28), 1933, 749.

or 3 sera from the patient, together with other controls of normal monkey serum. The serum-virus mixtures were incubated as described above, and finally inoculated intracerebrally in monkeys using 2, 3, or 4 monkeys for each serum-virus mixture. The total volume inoculated was usually 1.0 cc, but in a few instances 1.5 cc was used. Development of clinically observable paralysis and of typical pathologic lesions was used as a criterion of failure to neutralize. Monkeys not observed to have paralysis were considered negative and were not sacrificed for sections. Such monkeys were considered to have been "protected". Acute phase sera from the first 3 patients appeared to neutralize the virus. The tests were repeated and the results confirmed. However, in doing so one serum from each series was either used completely, or was lost through an accident. Thus serial dilutions of sera could not be performed, although it became obvious at this time that with antibodies present at the onset, such a method must be employed, if possible differences in titers were to be demonstrated. In the last 4 cases, serial 5-fold dilutions of serum were tested against the concentrated virus suspensions. In order to save monkeys, the sera were usually tested first in a 1:3 or a 1:5 dilution, since undiluted sera in earlier tests had always protected. Normal monkey serum controls were made at the lowest serum dilution used for the human sera.

*Results.* In the first 3 cases, children with spinal paralytic types of infection, ages 3 to 9, the undiluted acute phase sera taken on the 2nd, 4th and 6th days after onset neutralized the virus as did the convalescent sera. Not less than 4 monkeys were employed for each acute phase serum. Table I presents the results of the last 4 cases, showing age of patient, type of paralysis, number of days after onset of illness when each serum was drawn, and the paralysis ratio of monkeys inoculated. The first serum was frequently drawn before, or at the time of onset of paralysis, usually on the same day as the stool specimen from which the virus was isolated. This generally corresponded to the first or second day of hospitalization.

TABLE I. Monkey Neutralization Tests with Acute and Convalescent Phase Sera from Patients, Tested Against Homologous Viruses Isolated from the Same Patients.

| | | | | Patient's sera | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Acute | | | Convalescent | | |
| Patient and virus | Age of patient yr. | Type of paralysis | Results* monkey serum controls | Days after onset | Serum dilution | Results* | Days after onset | Serum dilution | Results* |
| A.K. | 6 | Spinal | 4/4 | 5 | Undil. | 0/2 | 74 | Undil. | 0/2 |
| | | | | | 1:5 | 0/2 | | 1:5 | 0/2 |
| | | | | | 1:25 | 2/2 | | 1:25 | 0/2 |
| | | | | | 1:125 | — | | 1:125 | 2/2 |
| | | | | | 1:625 | — | | 1:625 | 2/2 |
| G.P. | 16 | ,, | 3/3 | 4 | 1:5 | 1/2 | 24 | 1:5 | 0/4 |
| | | | | | 1:25 | 2/3 | | 1:25 | 3/4 |
| Camp. | 2 | ,, | 4/4 | 4 | 1:3 | 0/3 | 80 | 1:3 | 0/3 |
| | | | | | 1:15 | 3/3 | | 1:15 | 0/3 |
| | | | | | 1:75 | 1/2 | | 1:75 | 0/3 |
| Mo | 15 | bulbar | 4/4 | 4 | 1:3 | 0/3 | 45 | 1:3 | 0/3 |
| | | | | | 1:15 | 2/3 | | 1:15 | 0/3 |
| | | | | | 1:75 | 3/3 | | 1:75 | 0/3 |

* Numerator represents number of monkeys developing paralysis and the denominator the number inoculated.

*Discussion and conclusions.* In every instance the acute phase serum gave practically complete protection to all monkeys against a 20% monkey cord suspension which had usually been concentrated 10-fold by ultracentrifugation. A larger series of patients may show some without this titer of antibodies at, or before the onset of paralysis as did a group we reported who appeared to have been infected with a Lansing-like strain,[3] but among this group of 7 patients, ranging from 2 to 16 years of age, antibodies were definitely present either before infection or had already developed by the time of onset of paralysis.

The method of using serial 5-fold dilutions against a constant amount of virus, employed in the last 4 cases, appears to be satisfactory for this type of study and continued tests of this nature should afford important immunologic data on poliomyelitis. We realize that it would have been desirable to have known the titer of virus used in each instance, but we were influenced by consideration of the number of monkeys involved. It is obvious, however, from the results with serial serum dilutions, that the titer of virus used was neither in excess nor insufficient for the information sought.

Among the 4 cases in which serum titrations were made there is an apparent increase in antibody titer by the 45th, 74th and 80th days, but no definite rise in titer is apparent in the one case tested on the 24th day. In one instance the demonstrated increase in titer was only 5-fold, but in the others the increase was 25-fold or greater. It becomes apparent, therefore, that in poliomyelitis, as in most other virus infections, an increase in neutralizing antibodies usually develops as a result of infection. This finding encourages one to believe that a *type specific* serological test for this disease would have practical application. The frequent presence of specific antibody at readily detectable levels at the onset of paralysis or at the time of hospitalization lends further evidence against the possible usefulness of serum therapy, and possibly even serum prophylaxis. Obviously the results of these tests on man carry far more weight than tests on monkeys and chimpanzees following artificially induced infections.

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[3] Hammon, W. McD., and Izumi, E. M., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 424.

### 16683

## Hyaluronidase Inhibitors in Body Fluids in Normal and Disease States.

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(Introduced by W. J. Nungester.)

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Because of the probable relationship of hyaluronic acid to the formation and integrity of connective tissues, a relationship between hyaluronic acid metabolism and a large group of connective tissue diseases has been suspected, particularly in the case of rheumatic fever, rheumatoid arthritis, and disseminated vascular disease. The technical problems involved and the conflicting evidence which has been forthcoming have been extensively reviewed by Meyer.[1]

This report deals with attempts to relate this group of diseases to hyaluronidase activity, and, more significantly, with the apparent association of many malignant states in humans with disturbances of hyaluronidase inhibitors in the sera of such patients.

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* The Rackham Arthritis Research Unit is supported by a grant from the Horace H. Rackham School of Graduate Studies of the University of Michigan.

[1] Meyer, K., *Physiol. Rev.*, 1947, **27**, 335.

*Method.* The method used depends on the observation of Seastone[2] that capsules of a group C streptococcus isolated from guinea pigs consist largely of hyaluronic acid and that hyaluronidases from a variety of sources exert a destructive effect on the capsule which is a function of time and enzyme concentration.

This principle has been utilized previously by McClean[3] for *in vivo* studies. In a discussion of methods for hyaluronidase estimation Meyer[1] has stated that the decapsulation method is unsatisfactory because of spontaneous decapsulation of the test organism.

The technic as now modified has proved to be free of this objection. It offers a highly sensitive method for quantitative assay of hyaluronidase concentrations in the range between .001 and .0001 viscosity reducing unit per cc. Since turbidity or viscosity of the test materials do not interfere, the method is particularly adaptable to comparative studies of hyaluronidase inhibitors in serum and other body fluids in normal and disease states.

*The organism* is maintained by daily transfer on rabbit blood agar plates. Only well isolated, large (1 mm diameter) mucoid colonies are fished for subculture to agar or broth. After overnight incubation, these surface colonies may reach as much as 3 mm diameter, and are soft, mucoid, and hemolytic. Blood agar cultures over 24 hours old are not suitable for transplanting to serum broth.

A heavy inoculum, consisting of from 5 to 10 large well isolated, mucoid colonies, is transferred to a tube of Difco Brain Heart Infusion broth enriched with 10% sterile heated (56°C/20 minutes) hog serum. The culture is incubated at 37°C for 4 to 6 hours. After incubation the culture tubes are centrifuged at high speed for 5 to 10 minutes, and the sediment resuspended in 2 to 3 cc of M/15 phosphate buffer, pH 6.5. This is the indicator suspension of encapsulated organisms. It is to be emphasized that in a properly prepared suspension every streptococcal chain is heavily encapsulated.

As Seastone found, capsules are better preserved during drying and fixing in the presence of blood or serum. This step can be eliminated by coating slides with a thin coat of Mayer's egg albumen before dropping loopfuls of bacterial suspension on them. The slides are then dried in air and stained with Wright's stain by the usual technic.

*The enzyme* used in these studies was a dried testicular hyaluronidase assaying 230 VRU/mg, supplied to us by Drs. VonderHeide and Rodney of the Parke-Davis Research Laboratories. A single batch has been used throughout these studies. However, other preparations from the Schering Laboratories gave comparable results.

To study enzyme inhibition 0.2 cc of enzyme containing 1.0 VRU/cc and 0.1 cc of the serum or its serial dilutions were added to oven-dried 13X90 mm tubes. Racks were shaken and then 0.1 cc of organism suspension was added to the tubes. Inhibition was expressed as the highest dilution of serum inhibiting the decapsulation of the test organism for 90 minutes.

*Rheumatic Disease.* Guerra[4] has reported that salicylates are capable of inhibiting the spreading action of hyaluronidase in skin. Using concentrations of buffered sodium salicylate from 5 to 100 mg%, no inhibition of hyaluronidase was demonstrable. (Table I) Inasmuch as Meyer[5] has described a gentisic acid conjugate of salicylate in urine capable of inhibiting hyaluronidase, the urine and serum of patients taking large doses of salicylates were tested for hyaluronidase inhibitors.

TABLE I.
Inhibition of Testicular Hyaluronidase *in vitro*.
Each tube: Enzyme 0.2 cc 1.0 VRU/cc
Organism susp. 0.1 cc
Drug 0.1 cc

| | Conc. of drug mg % | | | |
|---|---|---|---|---|
| Sod. salicylate conc. | 100 | 50 | 25 | 5 |
| Inhibition obtained | 0 | 0 | 0 | 0 |
| Para amino benzoic acid conc. | | 50 | 25 | 5 |
| Inhibition obtained | | 0 | 0 | 0 |
| Gold sod. thiosulfate conc. | 1.0 | 0.5 | 0.25 | 0.1 |
| Inhibition obtained | 0 | 0 | 0 | 0 |

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[2] Seastone, C. V., *J. Exp. Med.*, 1939, **70**, 361.

[3] McClean, D., *J. Path. and Bact.*, 1942, **54**, 284.

[4] Guerra, F., *Science*, 1946, **103**, 686.

[5] Meyer, K., and Ragan, C., *Fed. Proc.*, 1948, **7**, 173.

TABLE II.
Inhibition of Testicular Hyaluronidase by Body Fluids Before and After Oral Sodium Salicylate.

| | Dilution of body fluid | | | |
|---|---|---|---|---|
| | 1:1 | 1:5 | 1:10 | 1:20 |
| Normal serum | + | + | 0 | 0 |
| Normal serum after sod. sal. | + | + | 0 | 0 |
| Normal urine | 0 | 0 | 0 | 0 |
| Urine after sod. sal. | 0 | 0 | 0 | 0 |

Time: 90 min.
Conc. of enzyme 1.0 VRU/cc.
+ = capsules present.

(Table II) In the case of urine no inhibitor capable of inhibiting 1.0 unit per cc of enzyme could be found, indicating that in therapeutically occurring concentrations salicylate conjugates presumably present in urine had no significant inhibitory effect on this reaction.

In serum, which normally contains hyaluronidase inhibitor, no increase could be produced by the ingestion of salicylates (Table II). These observations, therefore, confirm the observations of Pike[6] and Dorfman[7] and coworkers, who were unable to demonstrate hyaluronidase inhibition *in vitro* unless concentrations of salicylate of 2 g% were used.

Similarly, gold sodium thiosulfate and para aminobenzoic acid, drugs with an apparent influence on the course of certain connective tissue diseases, were without effect in producing enzyme inhibition when employed in therapeutically occurring blood serum concentrations. (Table I).

The presence of hyaluronidase inhibitor in normal serum has been described by several observers, but few quantitative observations have been made in disease states. Haas[8] described an inhibitor in the sera of normal and diseased individuals. However, there were no distinct differences between rheumatic fever and several other diseases listed. The inhibitor substance was about equally active against like amounts of pneumococcus type VI hyaluronidase and bovine testicular extract.

Friou and Wenner[9] were unable to find such inhibition of testicular hyaluronidase using the mucin clot prevention method, but found inhibition of streptococcal hyaluronidase in rheumatic fever, post-streptococcal convalescents as well as many normals. It is apparent from a perusal of this data that the inhibitor described was not the same as that which we have found, differing in the following characteristics: 1. The inhibitor described by Friou was not present in all sera, being absent from many normal sera. In our studies all sera have been inhibitory to some extent, although the variation among normals is marked. 2. The inhibitor was active against streptococcal hyaluronidase and inactive against hyaluronidase of testicular origin. In our studies the inhibitor was demonstrably active against testicular hyaluronidase as well as streptococcal enzyme. 3. The inhibitor was present in preserved sera as much as 10 years old and did not appear to decline in fresh sera on storage for 1 month. The inhibitor with which we deal is destroyed by heating to 56°C for 20 minutes and will disappear gradually in refrigerated sera within a few weeks. Inasmuch as the mucin clot prevention technic used by Friou requires relatively large amounts of substrate and enzyme, it is possible that the inhibitor present in all fresh sera toward testicular hyaluronidase was masked by its relative insignificance.

With these differences in mind, it is therefore proper to consider the testicular hyaluronidase - antihyaluronidase in a new relationship. As can be seen from the scatter diagram (Fig. 1) sera of various normal individuals of either sex vary widely in their ability to inhibit varying concentrations of testicular hyaluronidase. Since the same quantities of untreated serum, buffer, and bacterial substrate were present in each tube, these variations cannot be accounted for by variations in salt concentration. It is clear that any conclusions relative to the type of hyaluronidase inhibitors discussed here, in normal as compared to disease states must

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[6] Pike, R. M., *Science*, 1947, **105**, 391.

[7] Dorfman, A., Reimers, E. J., and Ott, M. L., Proc. Soc. Exp. Biol. and Med., 1947, **64**, 357.

[8] Haas, E., *J. Biol. Chem.*, 1946, **63**, 163.

[9] Friou, G. J., and Wenner, H. A., *J. Infect. Dis.*, 1947, **80**, 185.

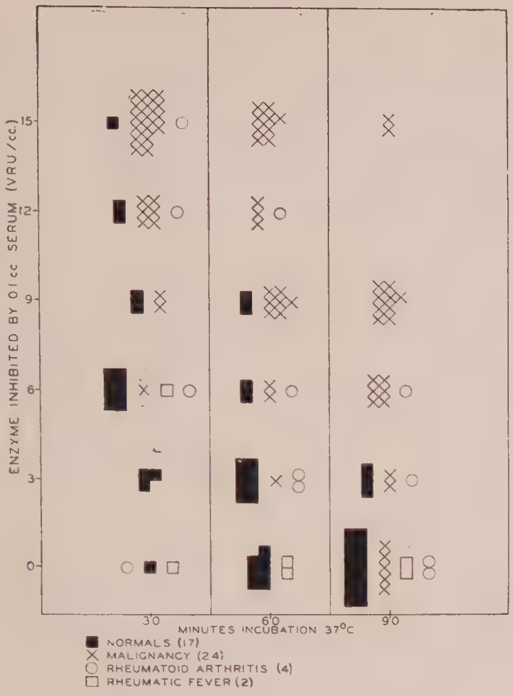

FIG. 1.

Relative testicular hyaluronidase inhibition by normal and disease sera.

take into account the wide range of normal variation.

When the sera of 4 active rheumatoid arthritis sera and 2 active and convalescent rheumatic fever sera were tested during the same test run with the group of 17 normals, they fell within the normal range. Because of the known instability of the inhibitor in stored sera, no attempt could be made to compare the sera of an active rheumatic fever patient with the same patient's convalescent serum weeks later. It seems evident that if changes do occur during the convalescence they are not outside the normal variation, which in itself remains unexplained. We, therefore, do not find evidence that either rheumatic fever or rheumatoid arthritis are typified by a clear cut disturbance in the particular hyaluronidase inhibitor with which this reaction is concerned.

*Malignancy.* Hyaluronic acid has been isolated from malignant tumors by Kabat[10] and by Meyer and Chaffee.[11] The presence of spreading factors (? hyaluronidases) in extracts of many animal and human tumors has been reported by Duran-Reynals.[12] The formation of spreading factor by malignant tissue has been regarded by Boyland and McClean[13] as the cause of the greater permeability of such tissue to nutrients.

On the basis of the unpublished observation of Rodney[14] that in Brown-Pearce carcinoma of rabbits a sharp rise in the titer of hyaluronidase inhibitor was detectable viscosimetrically in the sera of such animals, 24 sera of patients with a wide variety of malignancies both early and advanced and including leukemia and Hodgkin's disease were tested comparatively for hyaluronidase inhibitors with the same group of 17 control sera. As can be seen in the scatter diagram (Fig. 1), there is a statistical tendency for the cancer sera to show much greater inhibition under identical conditions. A few of the cancer sera show no characteristics which would distinguish them from normal sera, a few others are borderline, and some are markedly inhibitory. Since these observations have covered a relatively small group of normal, malignant, and other disease states, no conclusion can be drawn as to the exclusiveness of this property of excess inhibitor content for malignant sera, except to state that to date no other diseases studied have shown it.

Since testicular hyaluronidase was used throughout, to which no antibodies have been demonstrated, there is no evidence that an antigen-antibody reaction is involved, and the thermolabile nature of the inhibitor would suggest that it is not an antibody. The lack of differentiation between rheumatic states and normals would further suggest that the reaction is distinct from that described by Friou.

*Conclusions.* 1. A reliable biological method for the quantitative assay of small quantities of hyaluronidase or hyaluronidase inhibitor in the presence of body fluids is described.

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[10] Kabat, E. A., *J. Biol. Chem.*, 1939, **130**, 143.

[11] Meyer, K., and Chaffee, E., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 797.

[12] Duran-Reynals, F., and Stewart, F. W., *Am. J. Cancer*, 1931, **15**, 2790.

[13] Boyland, F., and McClean, D., *J. Path. and Bact.*, 1935, **41**, 553.

[14] Rodney, G., personal communication.

2. Presumably, the conjugates of salicylates as well as salicylate itself in therapeutic concentrations have no inhibitory effect on testicular hyaluronidase *in vitro*.

3. Thermolabile hyaluronidase inhibitor has been found increased in a significant proportion of the sera of human malignancies in a small series studied.

4. Rheumatic states do not differ from the normal with respect to this inhibitor toward testicular hyaluronidase.

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## 16684 P

# Calcification of Hypertrophic Epiphyseal Cartilage *in vitro* Following Inactivation of Phosphatase and Other Enzymes.*

J. WALDMAN. (Introduced by F. C. McLean.)

*From the Department of Physiology, The University of Chicago.*

Mineralization of hypertrophic epiphyseal cartilage *in vitro*, first demonstrated by Shipley,[1] requires concentration of calcium ions and phosphate ions in excess of a critical ion product, assumed to approximate the solubility product of a calcium phosphate salt. The concept of calcification solely as a precipitation phenomenon, however, fails to account for the specific localization of the mineral.

Robison[2] demonstrated phosphatase in hypertrophic epiphyseal cartilage and achieved calcification by addition of phosphoric ester in minute amounts to media of subcritical ion product. Robison[3] regarded the local factor as possibly enzymatic since calcification *in vitro* had not been observed in tissues heated beyond 60°C, nor after complete inhibition of phosphatase by various chemical agents.

Gutman and co-workers[4] demonstrated phosphorylative glycogenolysis in hypertrophic epiphyseal cartilage and have contended that calcification *in vitro* utilizes this enzyme system. On the other hand, McLean *et al.*[5] have found little correlation of anaerobic glycolysis with calcification.

The present study was undertaken to determine whether or not enzymes are necessary for *in vitro* calcification.

*Procedure.* Slices of epiphyseal cartilage were taken from the long bones of young rats made rachitic on a modified diet 2965 of Steenbock and Black,[6] and were subjected to varied preliminary treatment. The slices were then incubated approximately 18 hours at 37°C in isotonic media containing known amounts of calcium and phosphate at pH 7.4. Following incubation the tissues were washed in distilled water and stained with 2% silver nitrate. Experiments were designed to compare calcification in inorganic phosphate with calcification in phosphoric ester (sodium glycerophosphate). In addition, extracts of epiphyseal cartilage were prepared and were

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* This work has been supported in part by research grants from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service; from the Josiah Macy, Jr. Foundation; and from the Williams-Waterman Fund of Research Corporation.

[1] Shipley, P. G., *Bull. Johns Hopkins Hosp.*, 1924, **35**, 304.

[2] Robison, R., *Biochem. J.*, 1923, **17**, 286.

[3] Robison, R., and Rosenheim, A. H., *Biochem. J.*, 1934, **28**, 684.

[4] Gutman, A. B., Warrick, F. B., and Gutman, E. B., *Science*, 1942, **95**, 461.

[5] McLean, F. C., Lipton, M. A., Barron, E. S. G., and Bloom, W., unpublished data cited in The Biological Action of the Vitamins, University of Chicago Press; Chicago, 1942, p. 197.

[6] Hess, A. F., Weinstock, M., Rivkin, H., and Gross, J., PROC. SOC. EXP. BIOL. AND MED., 1929, **27**, 140.

analyzed for phosphatase activity by the method of Huggins and Talalay.[7]

*Results.* Incubation of slices in silver nitrate c. 1:1000 for one hour, followed by washing in distilled water for one hour, caused complete inhibition of calcification in phosphoric ester substrate. On the other hand, heavy calcification was observed when similarly treated slices were incubated in inorganic phosphate substrate. Untreated control slices calcified heavily in both media. Phosphatase activity of cartilage treated with silver nitrate was nil.

Similar results were observed when bichloride of mercury (c. $10^{-2}$ molar) was used as the inhibiting agent.

Incubation of slices in distilled water for one hour at constant temperatures ranging from 25° to 100°C (at 5° intervals) revealed that calcifiability in phosphoric ester substrate was lost above 50° to 55°C as determined by subsequent incubation. Similarly, phosphatase activity decreased as the temperature increased, and was nil in tissues heated above 60°C. On the other hand, when inorganic phosphate substrate was used, calcification occurred in all tissues including those that had been boiled. In tissues heated above 65°C calcification was found to depend on a high concentration of medium: thus it was observed in media containing 1 mM calcium and 20 mM phosphate per liter, but it was absent when the phosphate concentration was less than 10 mM per liter.

*Conclusions.* 1. Calcification of hypertrophic epiphyseal cartilage *in vitro* is intrinsically non-enzymatic. 2. The phosphorylase and phosphatase systems in hypertrophic epiphyseal cartilage may have supplementary roles in the calcification mechanism, but they are not essential to the localized deposition of bone mineral.

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[7] Huggins, C., and Talalay, P., *J. Biol. Chem.*, 1945, **159**, 399.

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### 16685 P

## Aureomycin in Treatment of Pneumococcal Pneumonia and Meningococcemia.*

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*From the Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard), Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston, Mass.*

Aureomycin is a new antibiotic which is active *in vitro* and in experimental animals against gram-positive and gram-negative bacteria and is also effective against experimental infections with rickettsias and with viruses of the psittacosis lymphogranuloma venereum group.‡ The findings in 4 cases of pneumococcal pneumonia and in one case of meningococcemia are presented here briefly. The bacteriologic methods are described elsewhere.[1,2]

The cases of lobar pneumonia were of moderate severity and a single lobe was involved in each instance. All were in males ranging in age from 16 to 65 years. Pneumococci were identified and typed from the

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* Aided by a grant from the United States Public Health Service.

† Research Fellow, American College of Physicians.

‡ Data on isolation, pharmacology and activity *in vitro* and in experimental infections have been presented by workers of the Lederle Laboratories Division of the American Cyanamid Company at a Conference on Aureomycin, New York Academy of Sciences, July 21, 1948. Additional clinical and laboratory observations by the authors and by other workers were also presented at that conference.

[1] Paine, T. F., Jr., Collins, H. S., and Finland, M., *J. Bact.*, 1948, **56**, 489.

[2] Collins, H. S., Paine, T. F., Jr., Wells, E. B., and Finland, M., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 174.

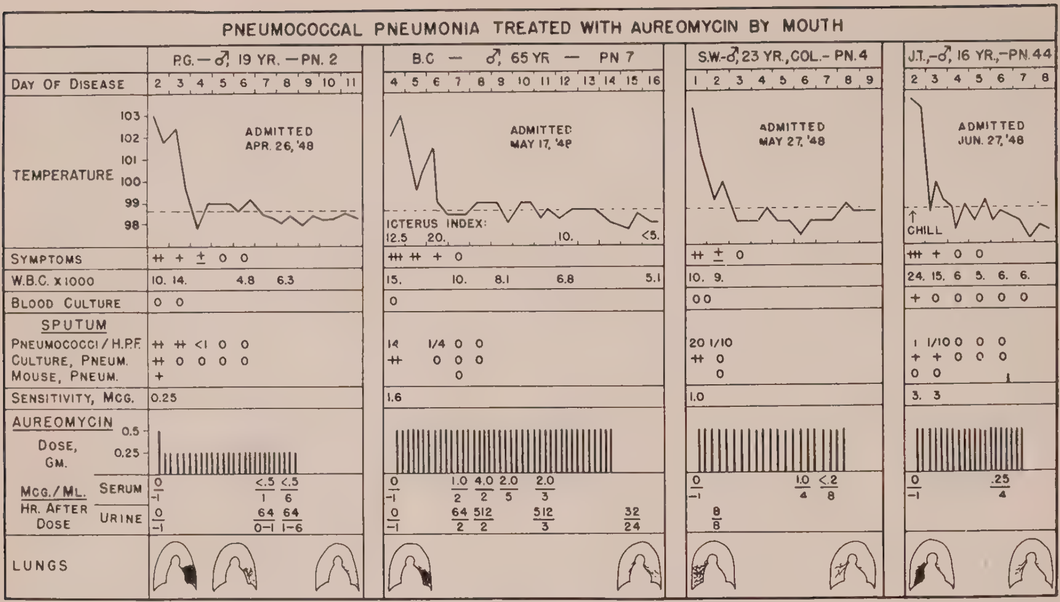

FIG. 1.
Relevant findings in 4 cases of pneumonia.

sputum in each instance and from the blood in one case before oral aureomycin therapy was started. One patient received an initial dose of 0.5 g followed by 0.25 g every 6 hours for a total of 5 g; the others received 0.5 g every 6 or 8 hours for a total of 10 g in 2 cases and 20 g in the oldest patient. No other chemotherapy or antibiotic was used. Some of the relevant findings are shown in Figure 1.

The patients became afebrile, and both subjective and objective improvement occurred in each instance between 18 and 36 hours after the first dose and was slowest in the oldest patients who had the severest illness and jaundice. The number of pneumococci decreased rapidly in the sputum following treatment and none could be demonstrated by any of the available methods, including mouse inoculation, after the second day of treatment. The pneumococci were completely inhibited by aureomycin in concentrations of 0.25 to 3.0 μg per ml.

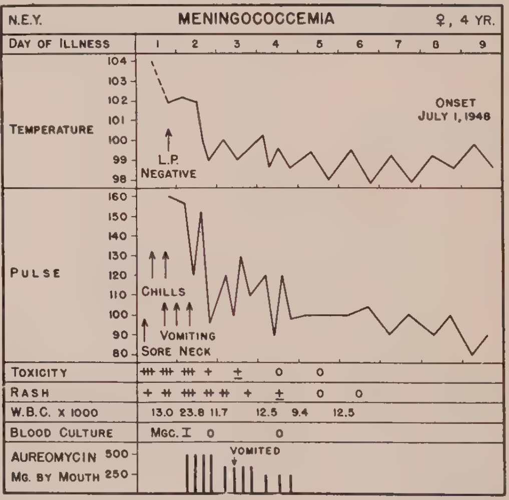

FIG. 2.
Relevant findings in a case of meningococcemia treated with aureomycin.

The relevant findings in the case of meningococcemia are shown in Figure 2. In this patient a tentative diagnosis of Rocky Mountain Spotted Fever was made on the basis of a history of tick bites, high fever, headache, slightly stiff neck and a rapidly spreading maculopapular eruption involving the trunk and extremities. Lumbar puncture yielded normal spinal fluid. Treatment was begun 12 hours after the onset of symptoms. The patient received 4 doses of 500 mg followed by 4 doses of 350 mg and then 3 of 250 mg, at 6-hour intervals. The patient was afebrile and much improved symptomatically and the rash had almost cleared within 18 hours after first dose. A blood culture obtained before treatment was started yielded meningococcus, group I and subsequent blood cultures were negative.

The results in these cases appear to be comparable to those obtained in similar cases treated with penicillin or with effective sulfonamides. There were no toxic effects attributable to aureomycin in any of these cases.

Further experience is needed to determine the optimum dosage and duration of treatment as well as the effect in more severe cases. The present findings, however, suggest that aureomycin may be an effective agent in pneumococcal and meningococcal infections and warrants further clinical trial in these and other types of infections.

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16686

## Excretion of Androgens and 17-Ketosteroids in the Cebus Monkey Following the Administration of Testosterone Propionate.

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(Introduced by H. Selye.)
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An increase in urinary 17-ketosteroids and androgens after administration of testosterone has been noted in man,[1] chimpanzee,[2] Rhesus monkey,[3] guinea pig[4] and rat.[5] Normally Cebus monkeys excrete minimal amounts of androgens as compared with other primates,[6] hence we wished to examine their 17-ketosteroids and androgen excretion after administration of testosterone propionate.*

*Material and Methods.* Six adult male Cebus monkeys, weighing 2.8 - 3.3 kg, were used. After a control period of 6 days, each animal received daily 50 mg of testosterone propionate subcutaneously, in 1 ml of peanut oil solution, for 12 days followed by 5 days during which no treatment was given. Throughout the period of observation (23 days) the urine of each monkey was continuously collected under toluene. Twice daily the urine specimen of all monkeys were pooled, acidified with 1% hydrochloric acid, placed in the refrigerator and extracted the following day. Hydrolysis, extraction and fractionation by Girard's reagent for chemical and biological assays was done as previously described.[7] The urinary extracts from the control period of 6 days (I), the treatment period of 12 days (II) and the post-treatment period of 5 days (III) were kept separate for chemical and biological assays. Colorimetric estimations were done by the Zimmermann method as modified by Callow *et al.*[8] and by the antimon trichloride technic.[9] Assays of androgenic activity were made using the chick comb method.[6]

*Results.* A. *Androgenic activity.* In a preliminary test we verified that the androgenic activity of the urine collected during periods I, II, and III was approximately the same. Therefore the final assay was done only on

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[1] Dorfman, R. I., and Hamilton, J. B., *J. Clin. Invest.*, 1939, **18**, 67.

[2] Fish, W. R., and Dorfman, R. I., *Endocrinology*, 1944, **35**, 22.

[3] Dorfman, R. I., and Hamilton, J. B., *Endocrinology*, 1939, **25**, 28.

[4] Dorfman, R. I., and Fish, W. R., *J. Biol. Chem.*, 1940, **135**, 349.

[5] Dorfman, R. I., *J. Biol. Chem.*, 1938, **123**, xxx.

[6] Valle, J. R., Henriques, S. B., and Henriques, O. B., *Endocrinology*, 1947, **41**, 335.

* Testosterone propionate was furnished through the courtesy of the Ciba Pharmaceutical Company of S. Paulo.

[7] Henriques, O. B., and Henriques, S. B., *Mem. Inst. Butantan*, 1946, **19**, 11.

[8] Callow, N. H., Callow, R. K., and Emmens, C. W., *Biochem. J.*, 1938, **32**, 1312.

[9] Pincus, G., *Endocrinology*, 1943, **32**, 176.

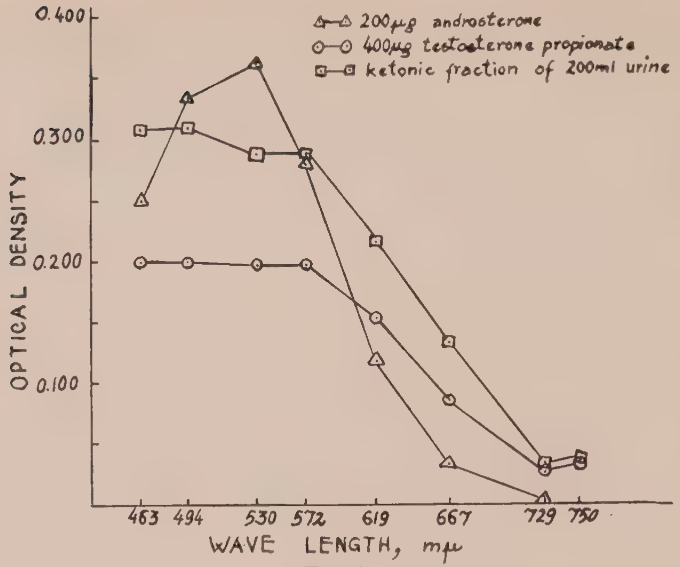

FIG. 1.

Comparison of absorption spectra of androsterone, testosterone propionate and ketonic fraction of urinary extract submitted to Zimmermann's reaction.

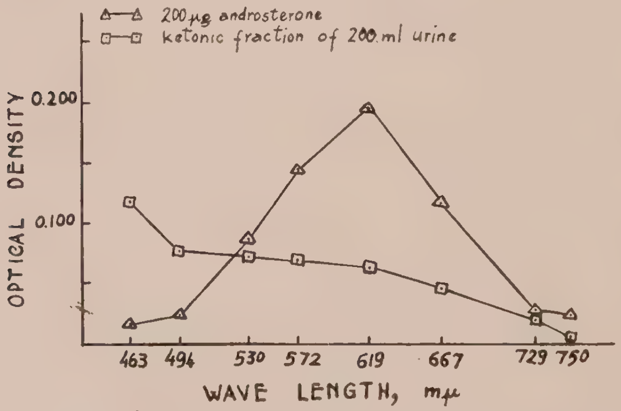

FIG. 2.

Comparison of absorption spectra of androsterone and ketonic fraction of urinary extract submitted to antimon trichloride reaction.

samples I and II. Androgen excretion was found to be 94.2 ± 24.5 μg per liter for sample I and 87.9 ± 39.0 for sample II. This corresponds to 10 and 6.5 μg per 24 hours, respectively, for one monkey.

B. *17-Ketosteroids.* Using Zimmermann's procedure, the color obtained was brownish instead of violet. Therefore we analysed this color with the Pulfrich photometer. Fig. 1 shows the absorption spectra of androsterone, testosterone propionate and the ketonic fraction from the extract of 200 ml of urine of untreated monkeys. Fig. 2 shows the absorption spectra of the same substances with the exception of testosterone, using the antimony trichloride reaction. It will be noted that there is no maximum absorption at 530 mμ for the urinary extract with the Zimmermann's reaction nor at 620 mμ with antimony trichloride procedure. After administration of testosterone propionate, no significant qualitative or quantitative change was detected in the absorption spectra.

*Discussion and Summary.* As previously stated, the administration of testosterone to many animals including man and the Rhesus monkey, results in an increased urinary excretion of androgens and 17-ketosteroids. In contradistinction to this we noted no increase in either of these substances in the urine of treated Cebus monkeys. This finding parallels those of Dingemanse and Tyslowitz,[10] Paschkis *et al.*,[11] who found no increase in the urine of dogs similarly treated. In dogs[11] and man[12] increased urinary estrogenic activity has been shown after testosterone treatment. Our results would suggest that in the Cebus monkey as in man and the dog, testosterone can be converted to estrogens, but further work is necessary to elucidate this point.

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[10] Dingemanse, E., and Tyslowitz, R., *Endocrinology*, 1941, **28**, 450.

[11] Paschkis, K. E., Cantarow, A., Rakoff, A. E., Hansen, L. P., and Walking, A. A., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 213.

[12] Dorfman, R. I., and Hamilton, J. B., *Endocrinology*, 1939, **25**, 33.

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## 16687

# Determination of Creatine, Creatinine, and Related Compounds in Urine by Means of Paper Chromatography.*

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*From the Research Laboratories of Distillation Products, Inc., Rochester, N. Y.*

A partition chromatographic procedure for the identification and estimation of creatine and creatinine and their demethylated analogues, glycocyamidine and glycocyamine (guanidoacetic acid) was devised during the course of an investigation of aminoaciduria in dystrophic humans.[1] Two different color-reactions were investigated to indicate the location of the various substances on the developed chromatogram. One was based on the Jaffe reaction, the formation of a reddish-orange product when alkaline picrate is added to solutions of creatinine and related compounds. This reaction has been used by Dent[2,3] to identify creatinine on a paper strip following partition chromatography. The other was a modification of the Voges-Proskauer reaction in which diacetyl in alkaline solution forms a pink product with creatine and similar compounds.

*Method Using Jaffe Reaction.* The Jaffe reaction is the conventional method for the detection of creatinine. It is dependent on a cyclic lactam structure such as is found in creatinine and glycocyamidine and is not given by the hydrated straight chain analogues, creatine and glycocyamine. It is necessary, therefore, to dehydrate the latter compounds *in situ* before applying the color reaction. It was found that heating the dry paper strips for one hour at 100-110°C transformed creatine to creatinine. Glycocyamine was dehydrated to glycocyamidine by heating for one hour at 100-110°C but the conversion was not quantitative. Further heating did not appear to increase the amount of glycocyamidine formed as evidenced by the depth of the color reaction. Therefore, in actual practice a constant heating period at a constant temperature was carefully followed in order to make valid comparisons between the depth of color formed with standard solutions and with urines of unknown composition.

Details of the experimental procedure are as follows: A 0.025 ml aliquot of urine or standard solution is placed dropwise at a starting point on a filter-paper strip. The strips are then developed for 15-20 hours using water-saturated solutions of butanol, phenol or lutidine-collidine (50-50) as solvents. The strips are air-dried, heated to 100°C for 1 hour and cooled to room temperature. The Jaffe reaction is obtained on spraying the strips with an alkaline picrate solution prepared by adding 10.0 ml of 10% NaOH to 50.0 ml saturated picric acid. The presence of creatinine or related compounds is evidenced by the appearance of an orange spot against a light yellow background. The color appears immediately and is comparatively stable. The strips are roughly evaluated in a manner similar to that suggested for amino acids.[1] The color intensity is estimated from "1" to "5" where "1" represents the faintest band observable and "5" represents an intense color. The *dw* value for each band is calculated by multiplying its width in cm by the color intensity. The procedure is quantitized by modification of any one of a number of published procedures: Extraction of the material and analysis by micro-Kjeldahl,[4] extraction of the colored product followed by conventional colorimetric analysis,[2,5] or less precisely by spot dilution technics[4] or by spot area technics.[6]

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* Communication No. 144.

[1] Ames, S. R., and Risley, H. A., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 131.

[2] Dent, C. E., *Biochem. J.*, 1947, **41**, 240.

[3] Dent, C. E., in Reifenstein, E. C., Conference on Metabolic Aspects of Convalescence, Josiah Macy, Jr. Foundation, New York, 1946, p. 126.

In order to test the accuracy and sensitivity of this procedure, the various compounds under consideration were analyzed individually, as well as in mixtures, and when added to urines. In Table I the average $R_F$ values obtained in the various solvents are given. Water-saturated solutions of butanol and lutidine-collidine give a better separation of creatine and creatinine than a water-saturated phenol solution. However, phenol gives an excellent resolution between glycocyamine and the others. Differentiation of creatinine and glycocyamidine by this method is not feasible because of the close correspondence of their $R_F$ values. The smallest amount of creatinine or creatine (converted to creatinine by heating) which could be detected was 11 μg. One hundred micrograms of glycocyamine heated to convert it to glycocyamidine yielded a barely distinguishable spot.

TABLE I.
Average $R_F$ Values for Creatine and Related Compounds.

| | Solvent ($H_2O$-saturated) | | |
|---|---|---|---|
| Substance | Butanol | Phenol | Lutidine-collidine |
| Creatine | .08 | .85 | .24 |
| Creatinine | .33 | .91 | .56 |
| Glycocyamine | .07 | .66 | .28 |
| Glycocyamidine | .30 | .89 | .52 |

Creatine and creatinine were Eastman Kodak Co. products. Appreciation is expressed to Dr. F. Smith of the University of Rochester Medical School for a generous sample of glycocyamine. Glycocyamidine in all cases was obtained *in situ* by dehydration of glycocyamine by heat. The solvents were used as water-saturated solutions at about 23°C and the chromatograms were developed in a thermostated room at the same temperature. The $R_F$ values are the averages of a large number of determinations using pure solutions, mixtures and samples of urine, and are reproducible to ±2 $R_F$ units.

The method for the determination of creatine and creatinine is therefore quite sensitive and precise. It is also easy to perform and it is possible to determine creatinine and related compounds in pure solutions or in urines with equal facility. Urines from a number of species, including rats, rabbits and humans, have been analyzed successfully. In studying urines it is possible to test for both amino acids and creatinine and related compounds on the same strip or sheet by spraying the Jaffe reagent onto the paper which has previously been treated with ninhydrin. The sensitivity and resolution of the creatinine determination is not impaired in the above procedure.

Following the completion of this investigation, a paper appeared by Maw[7] concerned with the determination of creatine by partition chromatography. In this procedure developed strips, after being airdried and heated in an oven at 100°C, were sprayed with 0.5 N sulfuric acid and heated at 100°C for 1 hour. This was stated to result in the dehydration of creatine to creatinine, the creatinine then being tested for by the Jaffe reaction. The method did not prove to be satisfactory in our laboratory, since the paper strips charred and were unusable. We have since learned[8] that Dr. Maw has been unable to satisfactorily reproduce this technic and is now following a procedure somewhat similar to ours with the exception that the strips are heated for 3 hours rather than 1 hour.

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[4] Polson, A., Mosley, V. M., and Wyckoff, R. W. G., *Science*, 1947, **105**, 603.

[5] Naftalin, J., *Nature*, 1948, **161**, 763.

[6] Fisher, R. B., Parsons, D. S., and Morrison, G. A., *Nature*, 1948, **161**, 764.

[7] Maw, G. A., *Nature*, 1947, **160**, 261.

*Method Using Diacetyl Reaction.* The use of alkaline diacetyl for the determination of creatine proved to be applicable to paper chromatography. This method is specific for creatine and gives no color with creatinine. Therefore, the strips were not heated, but with this exception the procedure as previously outlined was followed. A 50-50 water-alcohol solution saturated with sodium carbonate was prepared by adding 15 ml of absolute alcohol to 20 ml of saturated sodium carbonate solution and removing the resulting precipitate by filtration. Two ml of diacetyl were added to 10 ml of the resulting filtrate. When this solution was sprayed onto the strips, the presence of creatine was indicated by pink spots against a yellow background. Too high a concentration of sodium carbonate caused the strip to turn brown obliterating the pink color of the reaction. Insufficient sodium carbonate reduced the sensitivity of the method. After drying at room temperature the strips were placed in the oven at 100°C for exactly 5 minutes to hasten the color development which at room temperature is very slow. The smallest detectable amount of creatine was 33 μg, a sensitivity of about ⅓ that of the Jaffe reaction. Neither glycocyamine nor glycocyamidine in quantities up to 200 μg developed a color with the diacetyl reagent. The diacetyl reaction is given by several amino acids, and confusing results can be obtained on pathological urines. Its principal value is in the positive identification of creatine since it clearly differentiates it from creatinine.

*Summary.* A procedure for the identification and determination of creatine, creatinine, glycocyamidine and glycocyamine is presented. Following resolution by paper chromatography, the developed chromatograms are heated to 100°C for one hour to dehydrate creatine and glycocyamine *in situ* to creatinine and glycocyamidine respectively. The latter compounds and others of similar structure give an orange color on treatment with the Jaffe reagent (alkaline picrate).

The Voges-Proskauer reaction (alkaline diacetyl) for creatine was also investigated and found suitable but less precise.

$R_F$ values for creatine, creatinine, glycocyamine, and glycocyamidine are given for water-saturated solutions of butanol, phenol, and lutidine-collidine (50-50).

The chromatographic method possesses the advantage that creatine and creatinine can be determined independently and directly rather than by a differential procedure as in the conventional methods.

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[8] Maw, G. A., personal communication.

### 16688

## Biological Studies with Arsenic$^{76}$. I. Preparation of Arsenic$^{76}$ by Pile Irradiation of Cacodylic Acid.

R. L. Straube, W. B. Neal, Jr., T. Kelly and H. S. Ducoff.
(Introduced by L. O. Jacobson.)
*From the Biology Division of the Argonne National Laboratory, Chicago, Ill.*

Preliminary investigations into the production of radioactive arsenic, for distribution studies in animals and for clinical trial, indicated the need for inorganic radioactive arsenical compounds of a higher specific activity than could be obtained by pile irradiation of $As_2O_3$. This led to the consideration of the "Szilard Chalmers reaction."[1] Basically, this is a general reaction in which the recoil energy, imparted to the As nucleus upon neutron capture, ruptures the chemical bonds which hold it in the complex molecule, leaving it free and radioactive. D'Agostino[2] was able to separate arsenic$^{76}$ from cacodylic acid after bombardment by a radium-beryllium source, and Szilard[3] stated that pile irradiation of cacodylic acid would give good yields of radioactive arsenic. Preliminary experiments confirmed this.[4]

*Methods.* Cacodylic acid $(CH_3)_2AsOOH$, is a colorless solid that is easily soluble in water. It melts at 200°C and is extremely stable, being unaffected by fuming nitric acid, aqua regia, or potassium permanganate.[5]

An accurately weighed quantity (300-750 mg) of this substance, finely powdered, is introduced into a soft glass vial (8 x 40 mm) that has a small glass eyelet fused to its rounded bottom. The open end of the vial is then sealed with paraffin and, eyelet end up, placed within a larger quartz tube (12 x 125 mm). The latter is evacuated, sealed, and irradiated in a neutron pile.

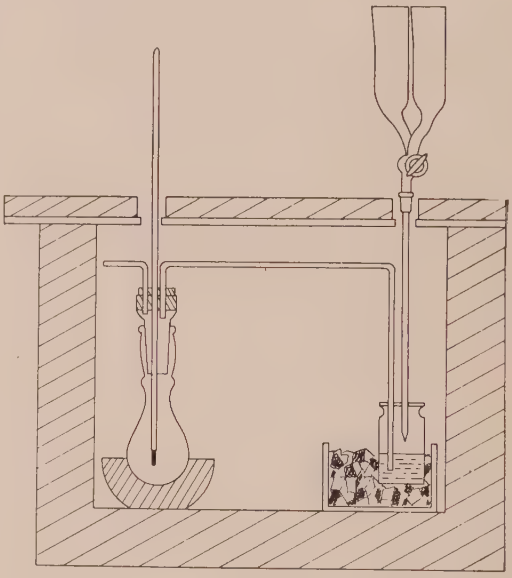

Fig. 1.
Cross section of lead shield with distilling apparatus in place.

Following irradiation, the quartz tube and its container are handled only by remote control apparatus and behind 10-15 cm of solid lead shielding. All chemical operations are carried on behind a U-shaped shield, having a floor and roof constructed of dovetailed, 10-cm thick, lead bricks, laid in alternating courses. A mirror, set at an angle at the open back of the shield, allows the operator to view the interior and the progress of the experiment. A cross section of the shield and the distilling apparatus it contains are shown in Fig. 1.

The irradiated quartz tube is slipped into a lead block placed behind the shield. The neck of the tube is then shattered by the remote control ratchet mechanism illustrated

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[1] Szilard, L., and Chalmers, P., *Nature*, 1934, **132**, 462.

[2] D'Agostino, O., *Gazzetta Chimica Italiana*, 1935, **65**, 1075.

[3] Szilard, L., personal communication.

[4] Brues, A. M., ed., Quarterly Report, Biology Division, Argonne National Laboratory, August 1, 1947, ANL-4078.

[5] Raiziss, G. W., and Gavron, J. L., *Organic Arsenical Compounds*, Chemical Catalog Co., Inc., New York, 1923.

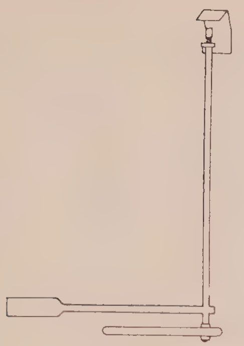

FIG. 2.
Remote control ratchet mechanism used in crushing the quartz capsule.

in Fig. 2. The "hot" cacodylic acid is transferred to the distilling flask with the aid of a right-angle hook that engages the eyelet on the bottom of the soft glass vial in which the acid is contained. The distilling vessel is an ordinary 100-ml Kjeldahl flask, the long neck of which is replaced by a 24-40 standard ground glass taper. The still head is the male portion of the taper, flared at the upper end to hold a No. 6, three-holed rubber stopper. A bent glass tube inserted through one hole admits compressed air, another holds the thermometer, and the third admits the end of the U-shaped distilling arm that leads to the receiving bottle.

For the usual sample of 500-750 mg of cacodylic acid, the distilling flask contains 7.5 cc of concentrated HCl (arsenic-free), and 0.4 g of cuprous chloride (CuCl). The cuprous chloride reduces any arsenic present in the pentavalent state to the trivalent form. Once the vial of cacodylic acid is added, the still head is sealed by manipulating the thermometer stem from above, through the roof of the shield. The flask is heated by a Glass-col* heating mantle and distillation is carried out to 110°C. Between 80-110°C the free trivalent arsenic is distilled as $AsCl_3$. The current of air, introduced through the still head, increases the yield. The distillate is caught in a graduated 30-ml bottle, containing 10 cc of cold, distilled water in a small ice-bath. The volatile $AsCl_3$ fumes, bubbling through the liquid, are hydrolyzed to arsenious acid. Neutralization to the phenolphthalein end point (pH 8.3) with 6 N NaOH follows. The NaOH is introduced through the long-stemmed burette that pierces the shield roof.

Aliquots for activity measurements and arsenic determinations are obtained with pipettes inserted in the remote control pipettor.[6] A 25-lambda micropipette withdraws a sample that is diluted in a 100-ml volumetric flask with 6 N arsenic-free nitric acid. Five cc of this solution is further diluted to the mark with nitric acid in another 100-cc volumetric flask. Four-cc samples of this final dilution are placed in duplicate Coors porcelain capsules and evaporated to dryness. These samples are then counted under a thin-window mica Geiger-Mueller tube and aluminum foil absorber of 54 mg/cm$^2$, using a conventional scaling and timer unit, and compared with a standard uranium source which is similarly mounted. Because of the high energy levels of the beta and gamma radiations of the arsenic sample ($\beta$ max $= 3.04$, $\gamma$ max $= 2.15$), mass absorption can be neglected.

The activity of the sample is calculated as follows:

$$\mu c\ As^{76}/ml = \frac{c/s\ sample}{c/s\ standard} \times \frac{\text{Absorption factor Al foil} \times \text{dilution factor}}{3.7 \times 10^{-4} \times \text{geometry factor of standard}}$$

The total amount of arsenic present, both stable and radioactive, is determined by means of the modified Gutzeit method for arsenic, as described in Scott's Standard Methods of Chemical Analysis.[7] Usually 0.1 cc of the solution obtained is sufficient to give a 20-30 $\mu$g stain. Knowing this value and the activity of the sample, the "specific activity", *i.e.*, the ratio of the number of radioactive arsenic atoms to the total number of arsenic atoms, is obtained.

*Results.* Table I is a summary of several

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* Obtainable from the Glass-col Apparatus Co., 1700 S. 7th Street, Terre Haute, Indiana.

[6] Tompkins, P. C., Broido, A., and Teresi, J. D., The Handling of Radioactive Materials in the Experimental Biology Section, Manhattan District Declassified Document (MDDC) 377.

[7] Scott, W. W., *Standard Methods of Chemical Analysis*, Van Nostrand Co., New York, 5th Edition, Vol. I, pp. 101-108, 1939.

TABLE I.
Yields of Radioactive Arsenic$^{76}$ from Pile Irradiated Cacodylic Acid (Szilard-Chalmers Reaction).

| Amt. cacodylic acid irradiated (mg) | NaCl % | Activity/cc (mc) | Arsenic/cc (mg) | Total vol. (cc) | Total activity (mc) | Specific activity (mc/mg) |
|---|---|---|---|---|---|---|
| 300 | 10 | 2.37 | 0.09 | 17.5 | 41.5 | 26.4 |
| 700 | 14 | 4.26 | 0.26 | 28.0 | 119.0 | 15.2 |
| 500 | 13.0 | 4.35 | 0.35 | 24 | 104.4 | 12.0 |
| 600 | 7.7 | 4.50 | 0.35 | 20 | 90.0 | 12.8 |
| 700 | 10.0 | 8.50 | 0.34 | 17 | 142.0 | 25.0 |
| 750 | 16.0 | 5.60 | 0.32 | 20 | 112.0 | 17.5 |
| 800 | 20.0 | 4.57 | 0.26 | 16 | 73.2 | 17.5 |
| 500 | 16.0 | 2.77 | 0.17 | 17 | 47.1 | 16.4 |
| 800 | 14.0 | 4.0 | 0.26 | 25 | 100.0 | 16.9 |
| 650 | 14.0 | 6.48 | 0.20 | 25 | 162 | 32.4 |
| 700 | 11 | 6.82 | 0.18 | 8 | 54.6 | 30.3 |

TABLE II.
Comparison of Specific Activities of Several Arsenic Compounds Exposed to Pile Irradiation.

| Material | $\frac{\text{mc As}^{76}}{\text{mg As}^{75}}$ | Enrichment |
|---|---|---|
| $As_2O_3$ | 0.5 | 1 |
| Cacodylic acid<br>$(CH_3)_2AsO(OH)$ | 15.0 | 30 |
| Arsenilic acid<br>$C_6H_4NH_2AsO_3H$ | 0.1 | 0.2 |

Values are representative ones.

preparations of arsenic$^{76}$ from pile irradiated cacodylic acid. There is wide variation in the specific activities because (1) time and power levels of pile irradiation were not uniform, and (2) activities were calculated to time of preparation of the arsenic solution.

It will be seen, however, that specific activities of arsenic$^{76}$ obtained by pile irradiation of cacodylic acid are considerably higher than those obtained by pile irradiation of arsenic trioxide. Table II compares specific activities following irradiation of cacodylic acid, arsenic trioxide, and arsenilic acid. In addition to the high specific activity of the resulting product, as can be seen from Table I, irradiation of cacodylic acid yields a solution of high total activity, this being a function of the high neutron capture cross section for $As^{75}$.

*Summary.* A method of obtaining $As^{76}$ of high specific activity by pile irradiation of cacodylic acid is described.

The assistance of Dr. Willard McCorkle, Mr. John Rose, and Dr. Alexander Langsdorf is gratefully acknowledged.

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## 16689

# Relation of Folic Acid and Vitamin A to Incidence of Hydrocephalus in Infant Rats.*

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Richardson and Hogan[1] observed hydrocephalus among infant rats and ascribed it to the inadequacy of the maternal diet. The diet, which was of the usual synthetic type, was supplemented with all of the recognized vitamins except pteroylglutamic acid, and the abnormality occurred in about 2% of the offspring. When the diet was supplemented with aqueous liver extracts, or with an eluate prepared from liver extracts, the abnormality was not observed. These concentrates would be expected to contain folic acid, but it was observed that crystalline "*L. casei* factor" would not cure the defect once it had developed. More recently Richardson and De Mottier[2] have produced hydrocephalus in an unrelated colony of rats.

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* Contribution from the Department of Agricultural Chemistry, Missouri Agricultural Experiment Station, Journal Series No. 1130.

This investigation was supported in part by a grant from the U. S. Public Health Service.

[1] Richardson, L. R., and Hogan, A. G., *J. Nutrition*, 1946, **32**, 459.

The purpose of this communication is to show the relationship of pteroylglutamic acid and vitamin A to the incidence of hydrocephalus among the offspring of rats that consume a synthetic diet.

*Methods.* Adult albino rats from the colony described by Richardson *et al.*[1] were placed on the experimental diets only a short time before mating since it was found that depletion periods of several weeks had no noticeable effect on the incidence of hydrocephalus. After pregnancy occurred the rats were transferred to individual round cages, containing wood shavings as litter, where the offspring were born. All of the offspring were allowed to remain with the dam until weaning. The young rats were observed frequently for abnormalities and were weighed at weekly intervals until the end of the fourth week, when they were weaned and discarded if no abnormalities occurred.

At the end of the second, third, and fourth weeks the rats were examined for symptoms of hydrocephalus by means of a light-transmission test. The head of the rat was placed over a small opening in a box containing an incandescent bulb, with the size of the orifice so adjusted that the head of a normal rat of comparable size transmitted no light. If transmission of light could be detected the animal was considered hydrocephalic, the severity being measured by the amount of light transmitted.

The basal synthetic diet was composed of acid-washed casein, 30%; cerelose, 52%; wood pulp, 3%; salts,[1] 5%; lard, 10%; vitamin A, 2000 I.U.; vitamin D, 285 I.U.; (Vitamins A and D were supplied in the form of oleum percomorphum, Mead Johnson & Co.) $\alpha$-tocopherol,[†] 2.5 mg; 2-methyl-1, 4-naphthoquinone, 2.5 mg; thiamine hydrochloride, 1.6 mg; riboflavin, 1.6 mg; pyridoxine hydrochloride, 1.6 mg; and calcium pantothenate, 4.0 mg. This diet was supplemented with various combinations of the following nutrients: Niacin, 5 mg; biotin, 0.020 mg; choline chloride, 100 mg; inositol, 100 mg; and ascorbic acid, 10 mg. Since none of these vitamins had any effect on the incidence of hydrocephalus, all of the rations containing any of these vitamins were grouped together and designated as Diet A. Diet A was supplemented with mcg of pteroylglutamic acid,[‡] 5% liver extract or 5% brewers' yeast. The vitamin A content of the diet was varied from 2000 I.U. to 100 I.U. per 100 g of diet.

*Results.* The data in Table I indicate that the addition of pteroylglutamic acid to Diet A markedly decreases the occurrence of hydrocephalus among the offspring.

During 1947 there were 1001 young born to dams maintained on Diet A and 10 or 1.9% of those weaned showed symptoms of hydrocephalus. Of the 1890 young from dams which received 50 mcg of pteroylglutamic acid per 100 g of diet only 2 developed the abnormality. During this same period 3 out of 1826 young rats born in the stock colony, which received a natural feedstuffs diet and served as a control, became hydrocephalic.

When it was observed that pteroylglutamic acid decreased the occurrence of hydrocephalus it was considered worthwhile to present all of our earlier data[1] as they have some bearing on the relation of pteroylglutamic acid to the

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[2] Richardson, L. R., and DeMottier, Jeanne, *Science*, 1947, **106**, 644.

[†] The $\alpha$-tocopherol, thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, calcium pantothenate, choline chloride, niacin, biotin, inositol, ascorbic acid, and 2-methyl-1, 4-naphthoquinone were supplied through the courtesy of Dr. D. F. Green, Merck and Co., Rahway, N. J.

[‡] The pteroylglutamic acid was supplied through the courtesy of Dr. T. H. Jukes, Lederle Laboratories, Pearl River, N. Y.

TABLE I.
Relation of Pteroylglutamic Acid in the Maternal Diet to Hydrocephalus in the Offspring.

| Experimental period | Supplements to diet A | No. of litters | No. born | No. weaned | Hydrocephalus No. cases | Hydrocephalus %* |
|---|---|---|---|---|---|---|
| 1947 | None | 157 | 1001 | 532 | 10 | 1.9 |
| | Pteroylglutamic acid (50 mcg) | 218 | 1890 | 1437 | 2 | 0.1 |
| | Stock ration† | 230 | 1826 | 1212 | 3 | 0.2 |
| 1944-1946 | None | 895 | 5026‡ | 2986 | 52 | 1.7 |
| | Liver extr.§ | 180 | 1197‡ | 937 | 0 | 0.0 |
| | Brewers' yeast | 83 | 488‡ | 309 | 3 | 1.0 |

* % of those weaned.

† A diet of natural feedstuffs, used as a control.

‡ The number of young retained, since in the earlier experiments the litters were reduced to 8.

§ The liver extract supplement consisted of either 5% of an aqueous extract of pork liver or 1% of a fullers' earth eluate of this extract.

TABLE II.
Relation of Vitamin A in the Maternal Diet to Hydrocephalus in the Offspring.

| Vitamin A content* | No. of litters | No. born | No. weaned | Hydrocephalus No. cases | Hydrocephalus %† |
|---|---|---|---|---|---|
| I.U. per 100 g | | | | | |
| 2000 | 51 | 335 | 226 | 5 | 2.2 |
| 1000 | 33 | 220 | 122 | 3 | 2.5 |
| 500 | 38 | 217 | 145 | 3 | 2.0 |
| 100 | 40 | 244 | 145 | 1 | 0.7 |

* The vitamin E content of the diet at each vitamin A level was varied from 0.5 mg to 2.5 mg but since the differences were of no consequence the data are combined.

† % of those weaned.

abnormality. From 1944, when the first case was observed, through 1946 there were 5026 young retained from dams which consumed diets that contained no added folic acid. Fifty-two animals, or 1.7% of those weaned, developed the syndrome while of the 1197 retained from dams which received liver extracts rich in folic acid, none showed symptoms. There were 488 offspring retained from dams which received 5% dried brewers' yeast in the diet and 1% of those weaned developed hydrocephalus. The folic acid content of the brewers' yeast is not known but it must have supplied some of the vitamin. It seems probable that the quantity was insufficient to prevent the syndrome.

The occurrence of hydrocephalus among young rabbits has been observed by Hyde[3] and by Greene,[4] and it was believed by Greene to be the result of a vitamin A deficiency. In view of the possibility that destruction of vitamin A might occur during storage of the rations, lower levels of vitamin A were fed in an attempt to increase the incidence of hydrocephalus. The results are shown in Table II.

The incidence of hydrocephalus was not increased by lowering the vitamin A content of the diet from the usual level of 2000 I. U. to as low as 100 I.U. per 100 g.

Although the incidence of hydrocephalus

TABLE III.
Moisture Contents of Rat Brains.

| Type Diet | Brains Analyzed No. | Moisture Content % |
|---|---|---|
| Stock | 23 | 79.93 |
| Synthetic | 23 | 79.91 |
| ,, | 6* | 88.10 |

* Brains from hydrocephalic rats.

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[3] Hyde, R. R., *Am. J. Hyg.*, 1940, **31**, 1.

[4] Greene, H. S. N., Department of Pathology, Yale University, personal communication.

TABLE IV.
Cerebrospinal Fluid Pressure Determined by Cisternal Puncture.

| Type Diet | No. of Observations | Cerebrospinal fluid pressure mm of water | Stand. dev. |
|---|---|---|---|
| Stock | 30 | 77 | 26 |
| Diet A | 39 | 67 | 29 |
| ” A | 3* | 220 | — |
| ” A + PGA | 34 | 78 | 29 |

* Rats showing symptoms of hydrocephalus.

on Diet A was 10 times as high as on the stock ration, it was still too low to permit rapid progress in discovering the cause. It was hoped that other less obvious symptoms of the deficiency might occur. Therefore a search was made for chemical and physiological differences between rats reared on synthetic and on practical rations. The moisture content of the brains from hydrocephalic rats is usually about 90%. That there was not a significant difference in moisture contents of brains of normal appearing rats from the synthetic and from the stock rations is shown by Table III. Twenty-three rats, 4 to 5 weeks of age, from each of the 2 types of rations were paired according to age, weight and sex, and their brains removed and dried for 24 hours at 65°C in a vacuum oven.

Hydrocephalus may be classified as internal or external depending on whether the fluid accumulates in the ventricles or in the subdural space and as communicating if there is free flow of liquid between the subdural space and the ventricles. In the communicating type there is a marked increase in cerebrospinal fluid pressure as determined by cisternal puncture.[5] Using the technic described by Griffith and Farris the cerebrospinal fluid pressure of the hydrocephalic rats was found to range from 150 to 350 mm of water. Thus the hydrocephalus appears to be the communicating type. Cerebrospinal fluid pressure determinations were made on rats, 4 to 6 weeks of age and weighing from 60 to 150 g, reared on the stock diet and on synthetic diets with and without added pteroylglutamic acid. The results are shown in Table IV. The variation within a group was quite large and there was no significant difference between groups.

*Summary.* About 2% of the young rats weaned from dams fed a synthetic type diet deficient in folic acid developed hydrocephalus. This abnormality could be largely prevented by the addition of pteroylglutamic acid to the maternal diet. Decreasing the vitamin A content of the diet to a low level did not increase the incidence of the hydrocephalus. There was no difference in either the moisture content of the brains or in the cerebrospinal fluid pressure of rats from the stock colony and those from dams receiving a synthetic type ration.

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[5] Griffith, J. O., and Farris, E. J., J. B. Lippincott Co., 1942, pp. 192-196.

## 16690

# Antiproteolytic Activity of Human Serum with Particular Reference to Its Changes in the Presence and Considerations of Its Use for Detection of Malignant Neoplasia.*

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(Introduced by S. C. Harvey.)

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Lacking a specific test for malignant neoplastic disease (cancer), it is possible that something short of this with a high degree of correlation, analogous to the serological test for syphilis, will be of value in selecting patients for more detailed examination than that feasible in the customary screening process of a "Cancer Detection Clinic." Many investigators have reported tests with this object in mind, without producing convincing evidence of a sufficient correlation with the presence or absence of malignant tumor. A review of these contributions will be undertaken in a later detailed presentation. Many of these tests are concerned with alterations in coagulation of the blood or its capacity to inhibit various enzymes. Another similarity is that they tend to give false positive reactions in the same types of pathologic conditions, other than malignant tumors.

These observations led us to believe that the basic mechanism might be an alteration in the proteolytic-antiproteolytic balance of the serum. Brieger and Trebing[1] suggested the use of the antifibrinolytic reaction of serum as a test for malignancy. Other reports by Von Bergmann and Meyer,[2] Herzfeld,[3] Roche[4] and many others were critically reviewed by Weil.[5]

This approach was discarded because of the false positive reactions and the subjective nature of the test and the fact that it was considered a cachexia reaction. Contributions to our understanding of the antiproteolytic response since that time have been reviewed by Grob[6] and Clark *et al.*[7]

A simple, reproducible, objective test of antiproteolytic (antitryptic) activity in serum was developed in this laboratory largely as a result of work by one of us (D.G.C.C.). This test is performed as follows.

*Materials.* 1. Veronal buffer: pH 7.4; Veronal 8.244 g; N/10 HCl 288 ml; Potassium oxalate .2948 g; NaCl 11.0 g; $H_2O$ to 2000 ml.

2. Trypsin:‖ A lyophilized pancreatic extract of bovine origin.

3. Fibrinogen:‖ A lyophilized material of bovine origin almost free from lysin.

4. Thrombin: A material prepared according to the method of J. H. Milstone,¶ from Armour beef plasma globulin ppt. pH 5.1 shipped in dry ice by air express. To 16 g of the Armour ppt. add 80 ml of veronal buffer. Grind well, as quickly as possible. Next add 0.1 N. NaOH with care to avoid an excess, to pH 7.4 (phenol red) and with alacrity in order to have as much as possible dissolved before the solution clots spontaneously. Centrifuge promptly at 2000 R.P.M. for 15 minutes,

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† National Research Council Fellow in Medical Sciences.

‡ Fellow, American Cancer Society, as recommended by the Committee on Growth of the National Research Council.

§ Jane Coffin Childs Fellow in Medical Research.

[1] Brieger, L., and Trebing, *Berl. klin. Woch.*, 1908, **45**, pt. 2, 1041, 1349, 2260.

[2] Von Bergmann and Meyer, *Berl. klin. Woch.*, 1908, **45**, 1673.

[3] Herzfeld, E., *Berl. klin. Woch.*, 1908, **45**, 2182.

[4] Roche, M., *Arch. Int. Med.*, 1909, **3**, 249.

[5] Weil, R., *Am. J. Med. Sc.*, 1910, **139**, 714.

[6] Grob, D., *J. Gen. Phys.*, 1943, **26**, 405.

[7] Clark, D. G. C., *et al.*, to be published.

‖ Obtained from Armour and Company, Chicago, Ill.

¶ We are indebted to Dr. Milstone for this method and for many other helpful suggestions.

TABLE I.
Example Test Report.

| Dilution | 1:50 | 1:60 | 1:70 | 1:80 | 1:90 | 1:100 | 1:110 | 1:120 | 1:130 | 1:140 | 1:150 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Test sera: | | | | | | | | | | | |
| A | +++ | +++ | +++ | +++ | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| B | +++ | +++ | +++ | +++ | +++ | ++ | 0 | 0 | 0 | 0 | 0 |
| C | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |

remove the supernatant and check to assure its pH of 7.4. Measure the supernatant and add 0.1 volume of .0275 M $CaCl_2$, stirring constantly until coagulation is complete. Discard the fibrin and store the solution in the refrigerator for use within one week.

5. Serum: Obtained from the patient's blood with special care that all glassware and needles used are clean and dry.

*Method.* The test should be run within 6 hours after the blood is drawn. The serum is prepared in dilutions of 1:50, 60, 70, 80, 90, 100, 110, 120, 130, 140 and 150. Solutions of trypsin, 35 mg to 100 ml of veronal buffer and fibrinogen 0.2 g to 40 ml of veronal buffer are prepared daily. The trypsin solution must be kept in the refrigerator. The thrombin is diluted 1:3 with veronal buffer and checked for activity as follows: to 0.1 cc of thrombin solution, 0.1 cc of fibrinogen and 0.3 cc of veronal buffer are added. A clot should form in 8 seconds. 0.25 cc of trypsin solution is added to 0.25 cc of serum dilution plus 0.3 cc of veronal buffer in 13 x 100 mm pyrex tubes. Allow to react 10 minutes at 25°C. Add 0.1 cc of fibrinogen, and allow to react 15 minutes at 36.5°C. Add 0.1 cc thrombin solution, and allow to react 15 minutes at 36.5°C. A constant temperature water bath is necessary. At this time the tubes are examined. The tube with the greatest dilution containing a definite clot is considered the end point. The end point is rarely in doubt, and in that case the test should be repeated in the doubtful range.

Using a large series of tubes it is necessary to add the reagents and read the results at specified intervals, usually 10 seconds, but trained personnel can cut the interval to 5 seconds with the desired accuracy. Results of repeated tests on the same serum within 6 hours or of samples from a normal individual on successive days will not vary by more than one dilution.

For the present each series of tests is run with a normal control serum, and the report is made with both the actual dilution titre and the ratio of the unknown titre to the normal serum titre. The test run shown in Table I may be used as an example. Serum A, the normal, is recorded as 80 or 100%, serum B would then be 100 or 125% and serum C >150 or >187%. The percentages so obtained have been used in the discussion of results.

*Discussion.* In view of the accuracy and objectivity of this method, it was considered desirable to explore again the possibility of its having significance for the detection of malignant new growths. Consequently, the antiproteolytic titre of the serum was determined on all new patients admitted to the "Tumor Clinic" as well as upon many in the hospital with known or questionable malignant tumors and with various diseases, not neoplastic. Blood was accepted from all sources without accompanying definitive information as to the patient's disease.

The data obtained were assembled and correlated and a method of statistical analysis** applicable to this problem was developed and applied. This analysis revealed that titres below 112% of the normal control could be considered as negative for malignant tumors with a possible error of 5%, and that titres above 136% of the normal control could be considered as indicative of malignant neoplasia with a possible error of 5% whereas

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** This was made by Mrs. Sylvia Johnson of the statistical division of the Tumor Registry with the assistance of Mr. David Votaw, Jr., of the Department of Mathematics of Yale University, to whom we are greatly indebted. The method employed will be reported elsewhere.

TABLE II.
Diagnosis.

| % of normal titer | Malignant neoplasia | Benign neoplasia | No apparent disease | Disease other than malignancy | Malignant neoplasm after definitive therapy | |
|---|---|---|---|---|---|---|
| | | | | | No recurrence | Recurrence |
| 75 negative | 1 | 5 | 5 | 7 | 2 | 0 |
| 87 ” | 4 | 8 | 6 | 4 | 1 | 0 |
| 100 ” | 3 | 45 | 68 | 48 | 42 | 0 |
| 113 doubtful | 10 | 15 | 15 | 22 | 14 | 1 |
| 125 | 10 | 4 | 1 | 7 | 5 | 0 |
| 137 positive | 7 | 0 | 1 | 1 | 0 | 0 |
| 150 ” | 13 | 0 | 0 | 0 | 0 | 2 |
| 165 ” | 15 | 0 | 0 | 0 | 1 | 1 |
| 175 ” | 14 | 0 | 0 | 0 | 1 | 3 |
| 187 ” | 15 | 0 | 1 | 1 | 0 | 2 |
| 200 ” | 16 | 0 | 0 | 3 | 0 | 0 |
| over 200 | 7 | 0 | 0 | 4 | 0 | 2 |
| | — | — | — | — | — | — |
| Total cases in each category | 115 | 77 | 97 | 97 | 66 | 11 |

All cases studied are listed according to the category of disease and the ratio of their test titer to the normal titer.

TABLE III.

| | Positive, % | Doubtful, % | Negative, % |
|---|---|---|---|
| Malignant neoplasm | 75 | 18 | 7 |
| Benign neoplasm | 0 | 25 | 75 |
| Patients without apparent disease | 2 | 17 | 81 |
| Patients with diseases other than neoplasia | 9 | 31 | 60 |
| Post-treatment patients: | | | |
| 1. Without recurrence | 3 | 29 | 68 |
| 2. With recurrence | 90 | 10 | 0 |

The percentages of patients in each group giving reactions in the positive, doubtful, and negative ranges are recorded.

with titres in the range between 112% and 136% of the normal control, the significance of the findings is doubtful. All patients with a definitive diagnosis on whom one or more tests had been made were reviewed. It is realized, of course, that complete certainty as regards the diagnosis will depend upon prolonged follow-up of these patients. 463 patients are included in this study, of whom 115 had malignant neoplasms, 77 had benign neoplasms, 97 were without apparent disease, 97 had demonstrable disease other than malignant or benign tumor and 77 had had malignant neoplasm for which they had received definitive therapy. From a study of Table II it is apparent that (a) no case of "benign neoplasia" gave a positive reaction, while 75% were negative and 25% were doubtful; (b) only two cases without apparent disease gave a positive reaction while 81% were negative and 17% were doubtful; (c) of the patients with diseases other than neoplasia 9% gave positive, 60% negative and 31% doubtful reactions; (d) on the other hand, of the patients with known malignant neoplasia, 75% gave positive reactions and only 7% were negative, while 18% were doubtful. This material is summarized in Table III.

The impression has been gained that after presumed complete removal of the malignant tumor or definitive therapy by x-ray the titre slowly falls to normal and does not rise again unless there be a recurrence. Of the patients without apparent clinical recurrence only 3% gave a positive reaction, as compared with 90% of those with clinical recurrence.

No detailed account of the false negative or false positive reactions can be given in this brief report. In general the false negatives fall into 3 classes: (1) patients with tumors of borderline malignancy; (2) patients with very small carcinomas; and (3) those with extensive terminal malignant disease, or rapidly spreading metastases. The false positives fall almost entirely into 3 groups: (1) patients with advanced tuberculosis; (2) patients who have been operated upon very recently; and (3) patients with active infections. Of the last, those in whom the streptococcus is the agent can be ascertained by the use of the streptococcal antifibrinolysin test (Christensen,[8] and Boisvert[9]). Reactions in the doubtful range occur frequently with serum from patients with other diseases (such as diabetes and lues) which gave false positive reactions with previous antifibrinolysin tests.

A non-specific antiproteolytic reaction has been outlined, the results of which when carefully analyzed give a high correlation between a positive test reaction and the presence of malignancy in the patient whose serum is tested. Certain false negative and positive reactions occur and careful study will be necessary to define these. It may be hoped that methods to exclude the effect of other specific antifibrinolytic factors, such as the antistreptokinase, will be developed with the ultimate goal of a specific reaction for malignancy. For the present, the reaction is of value as a screening mechanism with a degree of correlation warranting extremely careful observation in all patients with a consistently elevated titre.

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[8] Christensen, L. R., *J. Gen. Physiol.*, 1945, **28**, 363.

[9] Boisvert, P. L., *J. Clin. Invest.*, 1940, **19**, 65.

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### 16691

## Streptomycin-Induced Chlorophyll-less Races of *Euglena*.

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In the course of attempts to obtain with the aid of antibiotics pure cultures of algal flagellates and related colorless forms, it was noticed that after exposure to streptomycin (STM), normal green pure cultures of several strains of *Euglena gracilis*, used for orientation experiments, became colorless and remained so on serial transfer in light on STM-free media. The theoretical implications of this observation prompted further study.

A study was made of this bleaching as a function of (1) concentration of STM, (2) duration of exposure to STM, (3) exposure under proliferating and non-proliferating conditions, and (4) exposure to STM in light and darkness.

*Methods.* The organism used for most of these studies is classified as *Euglena gracilis* var. *bacillaris*.[1] It has the advantage over the widely used Pringsheim strain of growing rapidly up to 32°C, while the Pringsheim strain does not grow above 28°C. Illumination for growth was supplied by 3500°K white or 6500°K "daylight" fluorescent lamps, at intensity levels of 150 to 300 foot candles. This was sufficient for practical light saturation of moderately dense cultures.

Streptomycin solutions were compounded from crystalline salts and were sterilized by filtration through sintered glass.

It has been found previously in unpublished experiments that *E. gracilis,* and the other green and colorless euglenoids available in pure culture, were stimulated several hundred-fold by, or required absolutely, an unidentified growth factor present in relatively very high concentration in liver concentrates refined for parenteral treatment of pernicious anemia. It was thenceforth simple to devise reproducible media virtually devoid of utilizable carbon and energy sources ("substrate"), but which allowed very heavy growth in light or darkness when appropriate substrates were added. A medium used in initial studies of bleaching is shown in Table I. This medium, supplemented with good substrates, gives green cells only a slight advantage in light over colorless cells.

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* Haskins Laboratories.

† Sloan-Kettering Institute for Cancer Research. Present address: Hopkins Marine Station, Pacific Grove, Calif.

[1] Pringsheim, E. G., personal communication.

TABLE I.

| Basal Medium. | Concentration (%) |
|---|---|
| $KH_2PO_4$ | .1 |
| $K_2HPO_4$ | .1 |
| $NH_4NO_3$ | .1 |
| $MgSO_4 \cdot 7H_2O$ | .02 |
| $K_3$ citrate · $H_2O$ | .08 |

Liver concentrate (Lederle, 1 ml ≅ 3.3 U.S.P. units), 0.02 ml/100 ml.

Trace elements (mg %): Ca 2.0, Fe 0.3, B 0.02, Co 0.01, Cu 0.001, Mn 0.1, Mo 0.01, V 0.001, Zn 0.02.

pH 6.8-7.0. Media distributed 10 ml/25 ml Erlenmeyer flask.

Notes: 1. Citrate, added as a metal carrier,[2] is not utilized by *E. gracilis.*

2. The organism grows through a remarkably wide pH range, even in the presence of acetate or 0.25% Na butyrate. In later work the initial pH was 6.5.

3. Liver concentrates from 2 other commercial sources gave similar results.

*Results.* The effect of STM as influenced by concentration, substrates, and illumination was observed in the experiment summarized in Table II, using the medium of Table I. The observations on color refer to the macroscopic aspect of the cultures. *E. gracilis* strains normally become colorless when placed in darkness and become green again rapidly when restored to light. The following conclusions were drawn from the experiment of Table II and similar experiments:

1. STM reduced growth in light to a level equal to or lower than that in darkness.

2. Growth in darkness was unaffected. As a corollary, STM did not interfere with the utilization of externally supplied substrates.

3. Certain amino acids (alanine and glutamate) were good substrates for growth in darkness, although they accelerated growth in light only slightly; they were inferior however to certain lower fatty acids (represented in Table II by butyrate).

TABLE II.

Effect of Varying Concentrations of Streptomycin (STM) and of Various Substrates in Light and in Darkness. Growth expressed as cells/mm³

| | Light | | | | Darkness | | | |
|---|---|---|---|---|---|---|---|---|
| | No STM | STM 100 μg/ml | STM 1000 μg/ml | STM 5000 μg/ml | No STM | STM 100 μg/ml | STM 1000 μg/ml | STM 5000 μg/ml |
| No substrate | G<br>1300 | G<br>410 | W<br>8 | 0 | 8 | 10 | 13 | 51 |
| Na butyrate 0.15% | G<br>1900 | W<br>760 | W<br>480 | W<br>238 | 950 | 680 | 720 | 880 |
| Na H glutamate 0.3% | G<br>1950 | G<br>750 | W<br>13 | W<br>43 | 204 | 178 | 190 | 189 |
| *DL*-Alanine 0.25% | G<br>980 | G<br>300 | W<br>190 | W<br>132 | 360 | 420 | 320 | 410 |
| Glucose 1.0% | G<br>1380 | G<br>240 | W<br>24 | W<br>42 | 430 | 158 | 147 | 147 |

W—white, G—green. All cells in darkness were white.

In later experiments, a comparison was made of the action of STM upon cells grown under conditions permitting active proliferation as contrasted with non-proliferating cells. To cope with the possibility that STM-treated cells might acquire additional nutritional requirements, or that STM-dependent individuals might arise, the experiments were designed as follows: (a) Proliferating conditions were obtained by using a medium containing butyrate, glutamate, tryptic digest of casein (Sheffield's "N-Z-Case") 0.3%, solubilized liver (Wilson's fraction "L") 0.02%, and yeast extract (Difco) 0.04%. This portion of the experiment was conducted in light and darkness with varying time of exposure to a constant concentration of STM (100 μg/ml), and with varying concentrations of STM with constant time of treatment (15 days). Flasks were inoculated with one drop of a normal green culture. At the end of the experimental periods, 1-drop transfers were made into duplicate flasks containing the same medium, one set having 100 μg/ml STM, the other set without STM. The original cultures and the transfer cultures were then incubated in light for observation of the degree of bleaching. (b) Exposure to STM under non-proliferating conditions was obtained by first growing the cultures in light for 2 weeks in a substrate-free medium similar to that in Table I, thus making the cells wholly dependent on photosynthesis for energy and carbon. The cultures were then moved into darkness, and, after a 24-hour period to allow the dissipation of reserve food, treatment with STM was begun and carried out in darkness. At the end of the periods of exposure, 1-drop seedings were made as before into the complex medium, again with and without STM, and the cultures incubated in light. The salient observations from these experiments were:

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[2] Hutner, S. H., *Trans. N. Y. Acad. Sci., Ser. II,* 1948, **10**, 136.

1. There was a smoothly progressive bleaching of the cultures with increasing duration of exposure and increasing concentration of STM. The secondary cultures in STM-free media accurately reproduced the gross aspect which the primary culture had at the moment of transfer, if a small allowance be made for the slight selective advantage of the green cells in light.

2. There was no difference in the degree of bleaching between proliferating and non-proliferating cultures, either in respect to the necessary duration of exposure to STM or to the concentration of STM required. There was no difference in these respects between light- and dark-treated cultures under proliferating conditions.

3. There was no evidence of development of STM-dependency, even in cultures exposed for 2 weeks to 1000 μg/ml.

4. With STM constant at 100 μg/ml, an exposure of 1 to 8 hours was required for loss of roughly 50% of the color, and 4 or more days for apparently complete bleaching.

5. With constant time of exposure (15 days), 1 μg/ml gave roughly 50% loss of color, and bleaching was nearly complete with 40 μg/ml.

6. Preliminary microscopic examination of partly bleached mass cultures showed them to consist of both pale and of almost fully green individuals. However a few green cells had only 1-2 somewhat ill-defined chloroplasts instead of the normal number (about 10). Also, some green cells did not have chloroplasts of normal thickness. Aged white cells, derived both from primary treated cultures and from subcultures, accumulated an unusually large number of red granules as compared with aged untreated green cells or with cells of *Astasia*. The stigma and paramylon grains remained permanently unaltered in all colorless cultures.

Similar experiments on a restricted scale were carried out with *E. gracilis* var. *urophora* and the classical Pringsheim strain. Bleached cells of *E. gracilis* var. *urophora* have now been carried through 5 transfers in light on STM-free media, and *E. gracilis* var. *bacillaris* through 9, and the Pringsheim strain through 2, without the least resumption of color. Parallel tests on several species of *Astasia* showed that like *E. gracilis* in the dark they were not inhibited by 5000 μg/ml STM, the highest concentration tested.

*Discussion*. "Directed evolution" has so far been achieved only in bacteria, as in the interconversion of types of *Pneumococcus*[3] and of *Escherichia coli*[4] under the influence of specific nucleoproteins. The change to the non-chlorophyll condition (apochlorosis) described here falls rather into the special class of "loss mutations" ("pertes de fonctions") discussed by Lwoff.[5] That this change follows a well-established evolutionary path is suggested by the following considerations:

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[3] McCarty, M., Taylor, H. E., and Avery, O. T., *Cold Spring Harbor Symposia Quant. Biol.*, 1946, **11**, 177.

[4] Boivin, A., *Cold Spring Harbor Symposia Quant. Biol.*, 1946, **12**, 7.

1. Permanently colorless euglenoids similar to *E. gracilis* are abundant in nature, and are referred to the genus *Astasia*.[6] Some species of *Astasia* have a stigma like that in the colorless strains here derived from *E. gracilis*.

2. The euglenoids, as compared with other algae, have one of the richest arrays of colorless counterparts of green species.[5]

3. As already mentioned, the strains of *E. gracilis* tested all grow well in darkness when provided with suitable substrates in a good basal medium and permitted free access to atmospheric oxygen, indicating that the loss of photosynthesis does not here involve a profound alteration in metabolism.

4. Colorless euglenas have been reported to arise spontaneously in artificial culture (see [5,6] for references).

Sexuality is lacking in euglenoids and therefore it is impossible to determine here by the methods used for higher plants whether bleaching is a purely cytoplasmic phenomenon or involves a certain measure of conventional genic control—the classic problem of plastid autonomy. Another related question is whether bleaching, permanent or temporary, can be induced in forms which retain chlorophyll when grown in the dark.

In photosynthetic bacteria, as in blue-green algae, chlorophyll is not localized in plastids. Strains of purple bacteria (*Athiorhodaceae*) adapted to aerobic conditions, grow well in darkness aerobically, with retention of characteristic color, although the pigments are diminished in amount.[7] When 5 strains of purple bacteria, representing 5 species, were treated with STM, the sharp inhibition of growth was the same in light and darkness, and there were no obvious changes in color in partially inhibited cultures.

During the preparation of this paper, articles by von Euler and Bracco[8] were received. They found that seeds germinated in STM solutions developed into seedlings whose leaves showed chlorophyll deficiencies associated with colorless or structurally defective plastids. They did not report observations beyond the seedling stage.

The effectiveness in respect to *Euglena*-bleaching of portions of the STM molecule, and of other antibiotics, actinomycete and non-actinomycete in origin, is being studied; such data should determine whether the bleaching phenomenon may be used as a specific test for streptomycin, and should also furnish some indication as to the mode of action of STM.

*Summary.* When acted upon by streptomycin, certain strains of *Euglena gracilis* undergo a permanent loss of chlorophyll, without being inhibited in growth if good energy and carbon sources are supplied to make up for the loss of photosynthesis. The degree of bleaching is proportional to the degree of exposure to streptomycin. Proliferating and non-proliferating cells, and cells exposed in the light and in the dark, appear equally susceptible to bleaching.

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[5] Lwoff, A., L'Évolution Physiologique. Étude des Pertes de Fonctions Chez les Microorganismes. Paris: Hermann et Cie, 1943, pp. 308.

[6] Pringsheim, E. G., *Biol. Rev. Camb.*, 1948, **23**, 46.

[7] van Niel, C. B., *Bact. Rev.*, 1944, **8**, 1.

[8] Euler, H. v., *Arkiv f. Kem., Min., Geol.*, 1948, **25A**, 1; Bracco, M., and Euler, H. v., *Kem. Arb. II*, 1948, **10**, 1; Bracco, M., and Euler, H. v., *Kem. Arb. II*, 1947, **10**, 1.

### 16692

# Absorption of Aortic Atherosclerosis by Choline Feeding.*

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A previous report by the authors described the preventive action of choline against the development of atherosclerosis of the aorta in a series of rabbits fed cholesterol and choline simultaneously.[1] Steiner[2] found highly suggestive evidence that 0.5 g of choline daily caused some reabsorption of atheromatous lesions produced in the aortas of 6 rabbits by cholesterol feeding for 170 days. In 4 other rabbits similarly treated by Steiner, choline failed to cause significant aortic atheromatous reabsorption. The present study was undertaken to determine whether choline could effect reabsorption of aortic atheromatosis in a larger series of rabbits fed double the dose of choline heretofore employed and studied over twice as long a period of time.

Anitschkow,[3] Scarff,[4] and other have demonstrated that the characteristic atheromatosis of the aorta does not disappear after withdrawing cholesterol from the diet of rabbits. This atherosclerosis persists up to 815 days after cholesterol withdrawal from the diet, indicating that spontaneous reabsorption of the aorta does not occur.

Forty-four white male rabbits approximately 3 months old, who were litter mates were used in this study. These animals were divided into Groups A and B each containing 21 and 23 animals respectively. All the animals were individually fed 0.5 g of cholesterol daily with a basic diet of Purina Chow for 184 days. The cholesterol was mixed with the chow as prepared especially for this study by the Golden State Milling Co. of Downey, Calif. The rabbits took their feedings without difficulty and showed a gain in weight in each instance.

At the end of the 184-day cholesterol feeding period and the cholesterol feedings were stopped in all animals. The regular chow diet was then given to the 21 Group A animals for 185 days; the animals were then sacrificed. After the discontinuation of the cholesterol feedings 23 Group B animals were fed 1.0 g choline daily for 185 days mixed with the chow as above. The animals took these feedings without difficulty, gaining weight and showing normal activity in all cases. At the end of the 185-day period the animals were sacrificed.

At postmortem examination the grade of aortic atherosclerosis was described as O whenever no involvement of the aorta by atherosclerosis was grossly visualized or palpated by the examining fingers. Grade 1 was employed to describe a slight degree of atherosclerotic involvement of the intima of the aorta. Grade 2 described moderate atherosclerosis of the aorta. Grade 3 indicated marked atherosclerosis. Grade 4 described complete atherosclerotic involvement of the surface area of the aorta.

Table I, Group A—the 21 control rabbits fed cholesterol alone for 182 days then the regular diet for 185 days showed at autopsy aortic atherosclerosis of varying degree in each animal.

Table I, Group B—the 23 rabbits fed cholesterol alone for 182 days, then the regular diet with 1.0 g choline daily for 185 days showed aortic atherosclerosis at autopsy

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Appreciation is herewith expressed to Drs. Walter E. Macpherson and Harry A. Davis of the College of Medical Evangelists for their aid in this investigation.

[1] Morrison, L. M., and Rossi, A., to be published.

[2] Steiner, A., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 411.

[3] Anitschkow, N., *Verhandl. d. deutsch. path. Gesellsch.*, 1928, **23**, 473.

[4] Scarff, R. W., *J. Path. and Bact.*, 1927, **30**, 647.

TABLE I.
Atherosclerotic Involvement of the Aorta in Rabbits Fed 0.5 g Cholesterol Daily for 184 Days.

| Grade of involvement | 0 | 1 | 2 | 3 | 4 | Total affected |
|---|---|---|---|---|---|---|
| No. of rabbits | 0 | 2 | 3 | 5 | 11 | 21 |
| % of rabbits affected | 0 | 9% | 14% | 24% | 53% | 100% |

TABLE II.
Atherosclerotic Involvement of the Aorta in Rabbits Fed 0.5 g Cholesterol Daily for 184 Days, Then 1.0 g Choline Daily for 185 Days.

| Grade of involvement | 0 | 1 | 2 | 3 | 4 | Total affected |
|---|---|---|---|---|---|---|
| No. of rabbits | 17 | 4 | 2 | 0 | 0 | 23 |
| % of rabbits affected | 74% | 17% | 9% | 0 | 0 | 26% |

in 26% of animals. Seventy-four percent of animals were free of demonstrable aortic atherosclerosis.

*Conclusion.* Choline caused reabsorption of aortic atherosclerosis in the majority of rabbits whose lesions had been produced by cholesterol feeding.

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### 16693

## The Relationship of Heparin Activity to Platelet Concentration.

C. LOCKARD CONLEY, ROBERT C. HARTMANN AND JOHN S. LALLEY.
(Introduced by G. S. Mirick.)

*From the Department of Medicine, Johns Hopkins University and Hospital, Baltimore, Md.*

An increased susceptibility of thrombocytopenic blood to heparin has recently been reported by Allen and his associates.[1] These authors found that following the addition of a standard concentration of heparin to blood, more protamine sulfate was necessary to restore the normal clotting time in thrombocytopenic blood than was required by normal controls. They interpreted this observation as suggesting that in thrombocytopenia an increased amount of a heparin-like substance may be present in the blood. Studies in our laboratory[2] on 12 patients with hemorrhagic diathesis associated with thrombocytopenia in no instance revealed evidence of a circulating anticoagulant. It therefore seemed quite plausible that the increased susceptibility of thrombocytopenic blood to heparin may be a direct result of the decreased number of platelets present.

*Experiments.* Using silicone-treated apparatus and high speed centrifugation (12,000 to 14,000 RPM, 17,500 to 22,000 g's) at low temperature (4°C), platelet-free plasma was obtained from normal individuals without the use of anticoagulants. This plasma could be kept fluid in iced silicone-treated tubes for at least several hours. Platelet-rich plasmas were obtained by a similar technic except that the blood was centrifuged only at 3000 RPM (about 1300 g) for 5 minutes. Platelet counts were performed on these plasmas. These two plasmas were mixed in different proportions and varying amounts of heparin* added. Clotting times of these plasmas in glass tubes were then determined using a 3 tube method at 37°C. Results are shown in the table.

These results clearly indicate that the

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[1] Allen, J. G., Bogardus, G., Jacobson, L. O., and Spurr, C. L., *Ann. Int. Med.*, 1947, **27**, 382.

[2] Conley, C. L., Hartmann, R. C., and Morse, W. I., II, *Bull. Johns Hopkins Hosp.*, in press.

* Solution of heparin (sodium salt), Lederle Laboratories, Inc.

TABLE I.

Experiment 1.

Clotting Times of Plasma Samples Containing Varying Numbers of Platelets and Concentrations of Heparin as Indicated. The clotting times were determined simultaneously on samples derived from pooled plasma specimens. Clotting times of each of the 3 tubes are recorded.

| | Clotting times in minutes of 1.0 ml portions in glass tubes at 37°C | | | | |
|---|---|---|---|---|---|
| Heparin (mg per ml of plasma) | Whole blood (Platelets 190,000 per cmm) | "Platelet-rich" plasma (Platelets 420,000 per cmm) | "Platelet-free" plasma 90% "Platelet-rich" plasma 10% (Platelets 42,000 per cmm) | "Platelet-free" plasma 95% "Platelet-rich" plasma 5% (Platelets 21,000 per cmm) | "Platelet-free" plasma (Platelets 2 per cmm) |
| 0 | (1) 14 | (1) 12 | (1) 12 | (1) 12 | (1) 16 |
| | (2) 15 | (2) 12 | (2) 16 | (2) 18 | (2) 16 |
| | (3) 16 | (3) 14 | (3) 16 | (3) 18 | (3) 20 |
| 0.00025 | (1) — | (1) — | (1) — | (1) 10 | (1) 44 |
| | (2) — | (2) — | (2) — | (2) 24 | (2) 78 |
| | (3) — | (3) — | (3) — | (3) 28 | (3) Partial clot in 24 hrs. |
| 0.0005 | (1) — | (1) — | (1) 12 | (1) 60 | (1) No clots |
| | (2) — | (2) — | (2) 30 | (2) 89 | (2) in 24 hrs |
| | (3) — | (3) — | (3) 36 | (3) 98 | (3) |
| 0.001 | (1) 15 | (1) 15 | (1) 32 | (1) 38 | (1) No clots |
| | (2) 16 | (2) 16 | (2) 53 | (2) Partial | (2) in 24 hrs |
| | (3) 20 | (3) 20 | (3) 66 | (3) clots in 24 hrs | (3) |
| 0.01 | (1) 7 hrs | (1) 51 | (1) No clots | (1) No clots | (1) No clots |
| | (2) No clots | (2) 113 | (2) in 24 hrs | (2) in 24 hrs | (2) in 24 hrs |
| | (3) in 24 hrs | (3) Partial clot in 24 hrs. | (3) | (3) | (3) |

Experiment 2.

| | Whole blood (Platelets 210,000 per cmm) | "Platelet-rich" plasma (Platelets 448,000 per cmm) | "Platelet-free" plasma 90% "Platelet-rich" plasma 10% (Platelets 44,800 per cmm) | "Platelet-free" plasma 95% "Platelet-rich" plasma 5% (Platelets 22,400 per cmm) | "Platelet-free" plasma (Platelets 4 per cmm) |
|---|---|---|---|---|---|
| 0 | (1) 11 | (1) 4 | (1) 10 | (1) 10 | (1) 10 |
| | (2) 15 | (2) 8 | (2) 14 | (2) 14 | (2) 16 |
| | (3) 16 | (3) 10 | (3) 17 | (3) 16 | (3) 17 |
| 0.00025 | (1) — | (1) 10 | (1) 10 | (1) 19 | (1) 23 |
| | (2) — | (2) 14 | (2) 14 | (2) 37 | (2) Partial clots |
| | (3) — | (3) 24 | (3) 21 | (3) 60 | (3) in 24 hrs |
| 0.0005 | (1) — | (1) 11 | (1) 23 | (1) 14 | (1) Partial clots |
| | (2) — | (2) 20 | (2) 26 | (2) 35 | (2) in 24 hrs |
| | (3) — | (3) 28 | (3) 31 | (3) 56 | (3) No clot in 24 hrs |
| 0.001 | (1) 22 | (1) 11 | (1) 19 | (1) 41 | (1) Partial clot in 24 hrs |
| | (2) 24 | (2) 22 | (2) 54 | (2) 65 | (2) No clots in |
| | (3) 29 | (3) 26 | (3) 65 | (3) 90 | (3) 24 hrs |
| 0.01 | (1) 60 | (1) 41 | (1) No clots | (1) No clots | (1) No clots |
| | (2) No clots | (2) 93 | (2) in | (2) in | (2) in |
| | (3) in 24 hrs | (3) 138 | (3) 24 hrs | (3) 24 hrs | (3) 24 hrs |

TABLE I (Continued).
Experiment 3.

| | Clotting times in minutes of 1.0 ml portions in glass tubes at 37°C | | | |
|---|---|---|---|---|
| Heparin (mg per ml of plasma) | "Platelet-rich" plasma (Platelets 208,000 per cmm) | "Platelet-free" plasma 25% "Platelet-rich" plasma 75% (Platelets 156,000 per cmm) | "Platelet-free" plasma 50% "Platelet-rich" plasma 50% (Platelets 104,000 per cmm) | "Platelet-free" plasma (Platelets 8 per cmm) |
| 0 | (1) 5<br>(2) 6<br>(3) 7 | (1) 7<br>(2) 9<br>(3) 9 | (1) 5<br>(2) 9<br>(3) 12 | (1) 7<br>(2) 13<br>(3) 21 |
| 0.001 | (1) 14<br>(2) 24<br>(3) 32 | (1) 12<br>(2) 20<br>(3) 29 | (1) 16<br>(2) 27<br>(3) 41 | (1) 25<br>(2) 67<br>(3) 91 |
| 0.005 | (1) 24<br>(2) 52<br>(3) 81 | (1) 25<br>(2) 73<br>(3) 100 | (1) 36<br>(2) 52<br>(3) 83 | (1) No clots<br>(2) in<br>(3) 3 hrs |
| 0.01 | (1) 36<br>(2) 74<br>(3) 105 | (1) 44<br>(2) 108<br>(3) No clot in 3 hrs | (1) 91<br>(2) No clots<br>(3) in 3 hrs | (1) No clots<br>(2) in<br>(3) 3 hrs |

delaying or inhibitory action of heparin on the clotting of normal plasma is inversely related to the concentration of platelets present.

*Discussion.* The interpretations of several clinical tests for detecting hypo- and hypercoagulability of blood have been based on rather uncertain theoretical grounds. One of these is the test devised by Allen and his associates[1] for determining the existence of an increased amount of a heparin-like substance in thrombocytopenic blood. Our observations show that the results of their tests can be explained by the variation in the platelet concentration alone.

The "heparin tolerance test"[3] has been proposed as a means of measuring the tendency to intravascular clotting. Although this test has been used in clinical studies, the variables which determine its results have been to a large extent unknown. Our experiments suggest that one factor which may influence the results of this test is the number of platelets present.

In tests in which there is a minimal agitation of normal platelet-free plasma, the addition *in vitro* of heparin to a final concentration of 0.0005 mg per ml completely inhibits coagulation in *glass* tubes at 37°C . Normal platelet-free plasma to which no anticoagulant has been added invariably clots when transferred to glass tubes at 37°C. It therefore seems reasonable that the amount of active heparin present in normal plasma is of the order of magnitude of 0.0005 mg per ml or less.

*Conclusions.* 1. The concentration of heparin required to inhibit or delay coagulation is directly related to the number of platelets present.

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[3] de Takats, G., *Surg., Gyn., and Obs.*, 1943, **77**, 31.

2. The increased susceptibility of thrombocytopenic blood to heparin is a direct manifestation of the decreased number of platelets present and does not necessarily indicate the presence of a heparin-like substance.

3. One variable which may influence the results of the "heparin tolerance test" is the number of platelets present.

4. Our results suggest that the amount of active heparin present in normal plasma is very small and at least of the order of magnitude of 0.0005 mg per ml or less.

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### 16694 P

## Effects of Electro-Convulsive Shock on Pregnancy in the Rat.*

R. L. Bacon and H. E. Rosvold. (Introduced by Calvin P. Stone.)

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In an investigation of the effects of electroconvulsive shock on the maternal behavior of the white rat, Rosvold[1] found that females did not bear litters if shocked within 12 to 15 hours after copulation and once daily for 14 days thereafter. The rats received 45 milliamperes for 0.2 second through alligator clips attached to the ears. This strength of current invariably induced a complete tonic-clonic convulsion.

Gross examination at autopsy suggested that the fetuses were being resorbed. No histological study of the uteri, ovaries, or pituitaries was made in the original experiments. Accordingly a series of experiments was repeated in order to permit microscopic study of these organs under various conditions with respect to the number of shocks and the time of their administration. This preliminary report is based on the study of 24 healthy nulliparous females, about 100 days of age in which mating was observed in the evening and vaginal plugs found on the following morning. The animals were divided into 3 groups and shocked according to the following schedules:

Group I. Five animals shocked once, 12 hours after mating. Three controls mated at the same time, but not shocked.

Group II. Five animals shocked once daily for 6 days beginning 12 hours after mating. Three controls mated at the same time, but not shocked.

Group III. Five animals shocked once daily for 11 days beginning 12 hours after mating. Three controls mated at the same time, but not shocked.

All animals were sacrificed with ether on the eleventh day after copulation and the uteri, ovaries, and pituitaries removed at once and fixed in Bouin's fluid. Table I indicates the results observed at autopsy. The uteri of all the controls contained normal embryos of the 11th day of development.

In the two uteri of Group II which showed resorption of the embryos, the remnants of the implantation sites were located on the antimesometrial side of the uterus. In all of the animals of Group III the implantation sites were located on the mesometrial side of the uterus and were reduced to from 1/2 to 1/5 the size of those in the uteri of control animals.

For this preliminary study a randomly selected implantation site from each animal was sectioned serially at 10 $\mu$ and stained with hematoxylin and eosin. Sections of ovaries were stained with hematoxylin and eosin, and pituitaries with Mallory-azan.

In the uteri of the two animals of Group II in which the conceptuses were undergoing resorption, the deciduae were apparently normal and contained many giant cells, but

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* Supported by a grant from the Scottish Rite Fund, administered at Stanford University by Calvin P. Stone.

[1] Rosvold, H. E., Doctoral dissertation, Stanford University, 1948.

TABLE I.
Effects of Electric Shock on Rats Autopsied on the 11th Day of Pregnancy.

| Group No. | No. of animals | No. of shocks | Uterine contents | |
|---|---|---|---|---|
| | | | Normal | Resorbing |
| I | 5 | 1 | 5 | 0 |
| II | 5 | 6 | 3 | 2 |
| III | 5 | 11 | 0 | 5 |

Control animals are not included.

appeared in a condition to be expected at 7 or 8 days of pregnancy rather than at 11 days. Some proliferation was occurring as indicated by the presence of a few mitotic figures in each section. The embryos, however, were similar in stage of development to normal embryos of the 6th day but were disintegrating. In the uteri of all animals of Group III the entire decidua was disintegrating and no embryonic tissue could be found. The uterine lining was being reconstituted so as to expel the entire necrotic mass into the lumen. The uteri of the 3 unaffected animals contained normal embryos.

The pituitaries of all of the affected animals in Group II and III showed some degranulation of the acidophils and, although no measurements were made, the basophils appeared reduced in size when compared with those of the controls. The pituitaries of the unaffected animals did not show these changes. The ovaries of all the affected animals showed many degenerating corpora lutea and a few which were histologically normal, but smaller than those of controls. The ovaries of the unaffected animals were essentially normal.

*Summary.* A single electro-convulsive shock administered to 5 pregnant rats 12 hours after mating had no effect on the course of pregnancy. Of 5 rats shocked once daily for 6 days beginning 12 hours after copulation, on the eleventh day 2 showed some degranulation of pituitary acidophils, slight reduction in size of basophils, small or degenerating corpora lutea and degenerating retarded embryos in the uteri. All animals similarly shocked for 11 days, showed the same pituitary and ovarian changes, and, in addition, complete destruction of the fetuses with necrosis and sloughing of decidua into the uterine lumen.

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### 16695

## Effects of Acute Intermittent Anoxia Upon Urinary Volume, Specific Gravity and Chloride.

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(Introduced by J. P. Quigley.)
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Silvette[1] demonstrated a 300% increase in urinary volume of rats exposed to 282 mm Hg barometric pressure for 3-hour periods. Ten human subjects exposed by Armstrong[2] to simulated 12,000 ft. for 4 and 7 hours in a low pressure chamber excreted 100 to 300% more urine than at sea level and the urinary specific gravity fell as low as 1.016. Pincus and Hoagland[3] observed a polyuria in men exposed to reduced oxygen pressure. Burrill,

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[1] Silvette, H., *Am. J. Physiol.*, 1943, **140**, 374.

[2] Armstrong, H. G., Principles and Practice of Aviation Medicine, The Williams and Wilkins Co., Baltimore, 1939, p. 284.

[3] Pincus, G., and Hoagland, H., *J. Aviation Med.*, 1943, **14**, 173.

Freeman and Ivy[4] detected a slight increase in urinary volume and chloride content in men exposed to 18,000 ft. for 2-hour periods. Langley and Clarke[5] and Lewis *et al.*[6] reported a polyuria in unanesthetized dogs exposed for prolonged periods to 18,000 and 20,000 ft. altitude equivalents.

The present investigation was undertaken to determine the effects of severe anoxic conditions upon urinary volume, specific gravity and chloride excretion at various stages of the anoxic period. In the work of earlier investigators anoxic exposures have been made over several hours and in such experiments the initial urinary changes have been masked by changes of a different character occurring during a latter portion of the anoxic period.

*Methods.* Adult Long-Evans rats, male and female, were exposed to reduced barometric pressures in chambers described by Van Middlesworth *et al.*[7] and urine collections were made after the method of Silvette.[1] All exposures to reduced pressure were made at 258 mm Hg (equivalent 27,000 ft.) at 23-26°C. The method differed from those of earlier investigators in that samples were collected every hour. This necessitated returning the animals to sea level pressures for 3 minutes at the end of each hour, returning the animals to the chambers, and redecompressing for another hour. This procedure was repeated until an anoxic interval of 3-5 hours had accumulated.

Urinary specific gravity was determined by the falling drop method, in which a standard drop was allowed to fall through a 124 cm column of benzene and the falling time (range 11.0-13.0 sec.) was measured to within 0.02 sec. Chloride analyses were made on 1 cc of urine by means of the Volhard-Harvey technic.[8] Urine collections from control animals were made over 4-6 hour periods, and the average excretion per hour was taken as the control value. All animals were fed ad libitum, but water was usually withheld for 2 hours before each experiment. The manipulations associated with the changing of the environmental pressure failed to modify the urine volume in 12 control animals subjected to the reduced barometric pressure (258 mm Hg) for 2 minutes before and after the control period. The urine volumes were essentially the same (0.10-0.16 cc/100 g/hr) as those animals simply allowed to remain continuously at sea level. No rats were used more than once in a 4 week period; thus the possibility of acclimatization was avoided. Over 100 rats were used throughout the investigation.

*Results.* One hour exposure to 258 mm Hg barometric pressure resulted in a drastic increase in urinary output (Fig. 1A). This increase ranged usually from 3-11 times the normal value. Temperatures around 24°C and adult female rats usually tended to produce the greatest volume of urine although occasionally female animals exhibited minimal effects. Young adult male animals often produced only the lower volumes. After the polyuria of the first hour, the rate of urinary excretion decreased but still averaged above normal for the duration of the experiment.

The specific gravity of the abundant first hour urine usually ranged from 1.004 to 1.010 (Fig. 1B). After the first hour, the specific gravity also began to approach control values but did not attain the original level during the experimental period.

Urinary chloride was greatest in the most dilute, most abundant urine (Fig. 1C) and the chloride concentration was drastically reduced as the total solid content increased with continued anoxia. After 3 hours at these severe hypoxic conditions, the detectable chloride had almost disappeared from the urine.

One series of experiments was designed to determine the effect of the dehydration of the first hour exposure. Four groups of 3 rats each were given a saline solution at the end of each hour of altitude exposure. The same volume and chloride content as had been excreted in the previous hour urine collection

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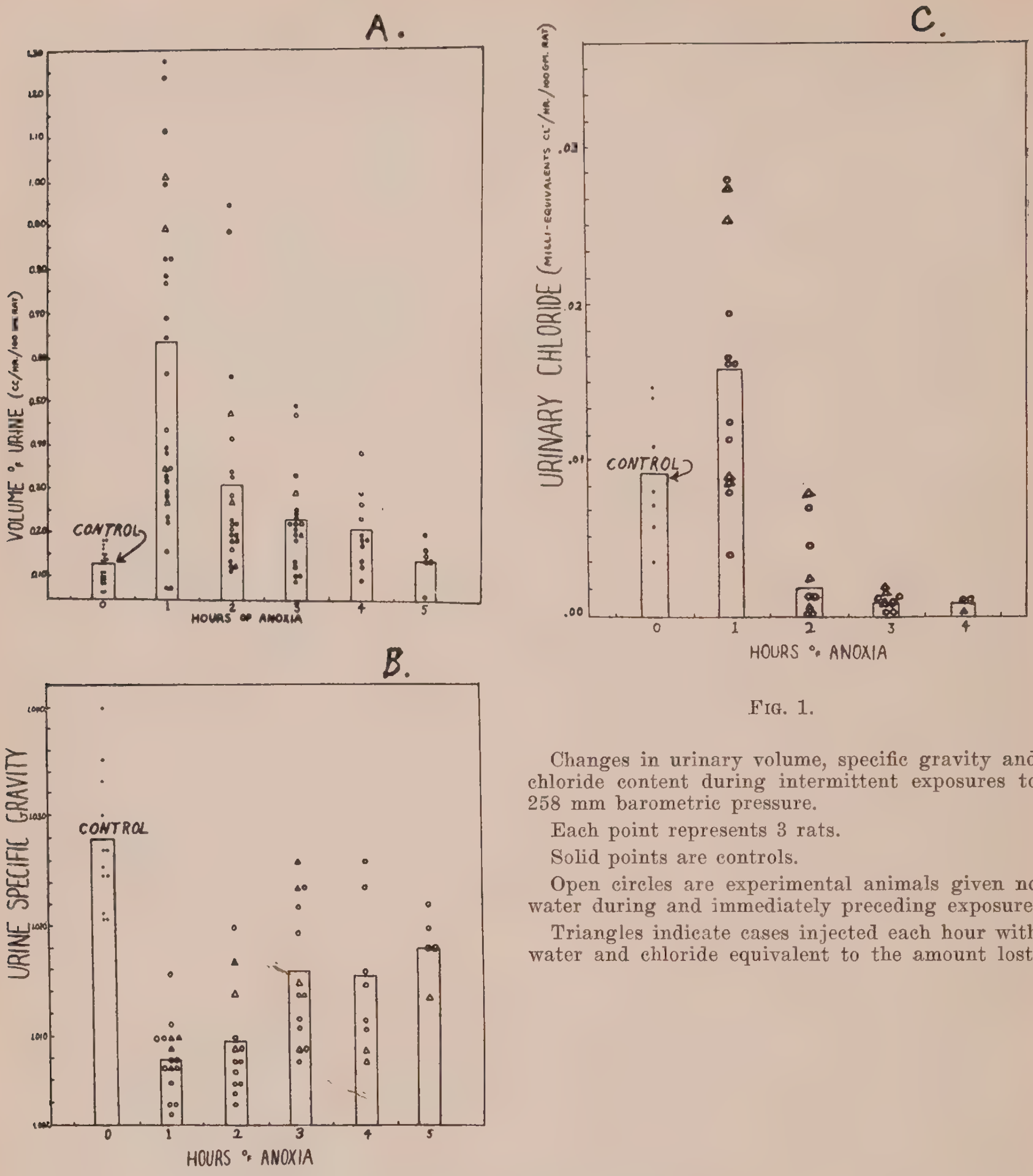

FIG. 1.

Changes in urinary volume, specific gravity and chloride content during intermittent exposures to 258 mm barometric pressure.

Each point represents 3 rats.

Solid points are controls.

Open circles are experimental animals given no water during and immediately preceding exposure.

Triangles indicate cases injected each hour with water and chloride equivalent to the amount lost.

was injected intraperitoneally. The subsequent urine volumes, specific gravity and chloride analyses are within the same ranges as the uninjected rats (triangle values in Fig. 1.) Apparently more comprehensive analyses for additional urinary components will be necessary for interpretation of the changes observed in urinary excretion during hypoxia.

*Summary.* The first hour exposure of rats to 258 mm Hg barometric pressure resulted in a 3 to 11 (average 7) fold increase in urine production. This increase in volume was accompanied by a marked fall in specific gravity (1.004 to 1.012, average 1.008). At the end of the third hour of intermittent anoxia the urinary volume and specific gravity approached normal sea level values. The chloride content, on the other hand, was *greatest* in the very dilute and abundant urine of the first hour and the chloride content decreased on repeated exposures until the more concentrated urine of the third hour contained practically no chloride.

## 16696

# Influence of Saliva upon Hemagglutination by Influenza Virus.*

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Study of the mechanisms involved in infection and resistance to influenza virus led to the demonstration of a virus-inactivating property in nasal secretions and in sputum.[1,2] The bulk of evidence points to the probability that the essential agency is specific antibody.[3] Rose[4] has continued further studies of human sputum, demonstrating wide variation in the capacity of different samples to neutralize influenza virus.

Recently several authors[5-8] have described the inhibition of the hemagglutinating action of influenza virus by carbohydrates and viscous materials. Although in an earlier study[2] saliva was not found to neutralize influenza virus in mice, the present study was undertaken to determine the effect of saliva upon hemagglutination since that fluid commonly contains a variety of substances similar in character to those shown to influence the action of virus upon erythrocytes *in vitro*.

*Materials and Methods. Collection and Treatment of Specimens.* Twenty to 25 cc of saliva were collected without chewing, in 50 cc graduated centrifuge tubes. Specimens were centrifuged at 2000-2500 r.p.m. for half an hour to remove solid particles and mucus because these substances were found to cause the chicken red cell suspensions to settle out on the bottom of the tubes in the same pattern as that seen with influenza virus and red cells. If supernatants were clear after centrifuging they were removed and used with no further treatment. However, when the specimens contained a great deal of mucus, further treatment was necessary. They were ground in mortars with alundum and centrifuged again. If still not clear and free of mucus, they were filtered through cotton and gauze. Even then small amounts of mucus remained in some specimens. It most cases the saliva was used the same day as collected.

*Virus Strain.* Allantoic fluid of the PR8 strain of influenza virus Type A which had 593 mouse passages and 71 egg passages was used in the majority of the experiments. In a few of the early experiments an eluate prepared from the 65th egg passage and allantoic fluid from the 70th egg passage of the same strain were used.

*Red Cell Suspensions.* From a stock 10% suspension of washed chicken erythrocytes, 0.25% suspensions were prepared daily. No cells more than 5 days old were used.

*Experimental.* 1. *Effect of saliva on PR8 strain of influenza virus.* Two-fold dilutions of virus were made in 0.25 cc volumes of physiological salt solution. To each dilution 0.25 cc of undiluted centrifuged saliva was added and mixed. This mixture stood at room temperature for 30-60 minutes before 0.5 cc of 0.25% chicken red cell suspension was added. Control titrations of virus and saliva alone were carried out under the same conditions.

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* This investigation was conducted with the aid of the Commission on Influenza, Army Epidemiological Board, Office of the Surgeon General, United States Army, Washington, D.C.

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The effect of saliva on the hemagglutinating titer of the PR8 strain of influenza virus was to decrease it from 8 to 512 times. The amount of inhibitor in saliva varied from person to person as well as from day to day in the same individual. Results of experiments with saliva from one individual on 6 occasions are shown in Table I. The results suggest that some deterioration of the inhibiting substance occurs on standing, even at 4°C.

2. *Inhibition tests with dilutions of saliva.* An inhibition test was performed employing a procedure similar to that described by Salk.[9] Two-fold dilutions of saliva were made directly in 0.5 cc of 0.25% red cell suspension. To the dilutions of saliva, 0.5 cc of a 1/1000 dilution of PR8 virus was added. This amount of virus constituted a final dilution of two agglutinating units in each tube.

This method of measuring inhibition by saliva, at first glance, did not seem to be as effective as that of the first experiments, owing perhaps to the fact that in the second type of test the saliva and virus do not stand together before the indicator (cell suspension) is added. Nevertheless, in terms of the hemagglutinating units inhibited in the two types of test the differences are not great. The results with several specimens are shown in Table II.

3. *Effect of saliva on chicken red cells.* From 3.5 to 4 cc of saliva were mixed with 8 cc of 0.25% chicken red cells and allowed to stand at room temperature for 30-60 minutes. The specimens were thoroughly shaken several times during that period. The cells were centrifuged and resuspended in 8 cc of fresh salt solution without washing. These cells were then added to a series of virus dilutions. A cell control of 0.5 cc salt solution and 0.5 cc of cells was included.

It was found that when a specimen of saliva caused spontaneous agglutination of red cells, the cells continued to agglutinate even though removed from the mixture and resuspended in fresh salt solution. In most cases treating cells with saliva had very little effect on the virus titrations. Thus the influence of the inhibitor appears to be upon the

TABLE I.
Effect of Saliva from One Individual Upon Hemagglutination by the PR8 Strain of Influenza Virus.

| Specimen | Experiment | Date of test | Age of saliva, days | Final dilution of virus 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 | 1024 | 2048 | 4096 | 8192 | 16,384 | Titer of virus | Aggl. units of virus inhibited |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 0.5 cc virus dil.<br>+<br>0.5 cc cell suspension | With all tests | | + | + | + | + | + | + | + | + | + | + | + | 0 | 0 | 4096 | |
| E.M. | 0.25 cc virus dil. | 1–22–48 | 0 | + | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 8 | 256 |
| | + | 27 | 0 | + | + | + | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 32 | 64 |
| | 0.25 cc saliva | 2–23 | 6 | + | + | + | + | + | + | + | + | 0 | 0 | 0 | 0 | 0 | 512 | 4 |
| | + | 3– 2 | 1 | + | + | + | + | + | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 128 | 16 |
| | 0.5 cc cell suspension | 3 | 1 | + | + | + | + | + | + | + | 0 | 0 | 0 | 0 | 0 | 0 | 256 | 8 |
| | | 5 | 0 | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 512 |

+ = Agglutination.
0 = No agglutination.

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TABLE II.
Effect of Saliva upon Hemagglutination of PR8 Strain of Influenza Virus as Indicated by the Inhibition Test.

| Specimen | Date of test | Age of saliva in days | Inhibition titer of saliva | Aggl. units of virus inhibited |
|---|---|---|---|---|
| B.B. | 2–23–48 | 6 | < 4 | 0 |
| | 26 | 0 | 32 | 64 |
| | 3– 2 | 1 | 64 | 128 |
| | 3 | 1 | 32 | 64 |
| | 29 | 0 | 32 | 64 |
| A.Z. | 17 | 0 | 8 | 16 |
| J.Q. | 17 | 0 | 8 | 16 |
| E.M. | 2–23 | 6 | < 4 | 0 |
| | 3– 2 | 1 | < 4 | 0 |
| | 3 | 1 | 4 | 8 |
| | 5 | 0 | 32 | 64 |

TABLE III.
Lack of Effect of Saliva Upon the Agglutinability of Chicken Red Cells.

| Specimen | Experiment | Date of test | Age of saliva in days | Titer of virus | Aggl. units of virus inhibited |
|---|---|---|---|---|---|
| Control | 0.5 cc<br>virus dil.<br>+<br>0.5 cc<br>normal cells | With all tests | | 4096 | |
| B.B. | 0.5 cc<br>virus dil.<br>+<br>0.5 cc cells<br>saliva-treated | 1-28-48 | 0 | 1024 | 2 |
| | | 2-23 | 6 | 2048 | 1 |
| | | 26 | 0 | 2048 | 1 |
| A.Z. | ” | 26 | 0 | 4096 | 0 |
| J.Q. | ” | 28 | 0 | 4096 | 0 |
| E.M. | ” | 22 | 0 | 256 | 8 |
| | | 27 | 0 | 512 | 4 |
| | | 2-23 | 6 | 2048 | 1 |

virus rather than upon the erythrocytes. The results shown in Table III illustrate the general lack of effect as well as the exceptional experience with saliva of E.M.

4. *Effect of heating saliva and virus.* Samples of saliva or virus were heated at 56°C for 30 minutes. All various combinations of the above tests were tried:

(a) Constant amount of heated saliva added to serial dilutions of unheated virus.

(b) Inhibition test—serial dilutions of heated saliva with constant amount of unheated virus.

(c) Unheated saliva added to heated virus.

(d) Inhibition test—unheated saliva with heated virus.

(e) Heated saliva added to heated virus.

(f) Inhibition test—heated saliva with heated virus.

One effect of heating the saliva was the elimination of spontaneous agglutination of red cells by certain specimens. In only one case did heating the saliva fail to eliminate this influence. Otherwise the heating of virus or saliva exerted no significant effect upon the action of saliva, illustrating that in this respect the inhibitory influence of saliva differs from that of the normal serum inhibitor.

*Summary.* Saliva of human adults possesses the capacity to inhibit agglutination of erythrocytes by influenza virus. Under the conditions of study the effectiveness varies among individuals and at different times. Its action is not significantly affected by heating at 56° C for 30 minutes; nor is its behavior influenced by heating of the virus. Nevertheless, the action of the inhibitor appears to be upon the virus rather than upon the erythrocytes. The nature of the inhibitor has not been determined but it is suggested that saliva represents a physiologic source of materials such as have been shown to interfere with hemagglutination in *in vitro* systems.

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16697

## Alteration of *Pasteurella pestis* Bacteriophage Following Successive Transfer on *Pasteurella pseudotuberculosis* and on Shigellae.

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A previous report[1] has described the activity of a strain of *Pasteurella pestis* bacteriophage which was able to lyse 19 of 27 strains of *Pasteurella pseudotuberculosis* and 6 of 37 strains of shigellae. By repeated transfer on *P. pseudotuberculosis* (Spokane strain) this phage became adapted so that it lysed all 27 strains of this species in high dilution. After transfer on shigellae, the phage increased its potency for sensitive strains of this genus. The present report describes the effect of successive transfer of the same *P. pestis* phage on *P. pseudotuberculosis* and on certain species of shigellae in various combinations.

The methods used in this study have been recorded.[1] All tests were incubated at 37°C. The transfers of phage were made by adding 0.5 ml of the previous passage to 10 ml of a broth culture of bacteria about two hours old and incubating until clear. After about 5 transfers no secondary resistant growth appeared and filtration of the lysate was unnecessary. The "purified" *P. pestis* phage previously described was used as a starting point in this study. The bacterial cultures were the same as those studied in the first report. Thirteen additional strains of *P. pseudotuberculosis* were investigated, of which all but one were lysed by the original phage.

In the first experiment, the *P. pestis* phage was transferred 22 times on *P. pseudotuberculosis* (Spokane strain) and then five times on each of the following in succession: *S. dysenteriae* (No. 44), *S. paradysenteriae* (type 103 No. 12), *S. sonnei* (No. 6), and *S. ambigua* (No. 33). It was found that any significant change occurred within 5 transfers on a given culture and that further transfers up to 25 made no difference. After each series of transfers, the adapted phages were tested on agar plates against each of the organisms listed and against *P. pestis*. Representative results are shown in Table I.

The results after transfer on 2 or 3 of the species of shigellae were essentially the same as those after transfer on all 4 species. The right hand column in Table I represents the effect of successive transfer on all 4 shigellae. It is noteworthy that after passage first on *P. pseudotuberculosis* and then on *S. dysenteriae,* the phage no longer lysed *P. pseudotuberculosis*.

A second series of experiments was planned to show the effect of successive transfer on

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TABLE I.
Highest Dilution of Original and Adapted Phages Producing Lysis of Plate Cultures of Various Organisms.

| | | Original *P. pestis* phage adapted to | | |
|---|---|---|---|---|
| Organism tested | Original *P. pestis* phage | PTB | PTB then *S. dysenteriae* | PBT then *S. dysenteriae, paradysenteriae, sonnei,* and *ambigua* |
| *P. pestis* | $10^{-7}$ | $10^{-7}$ | $10^{-7}$ | $10^{-6}$ |
| PTB | — | $10^{-7}$ | — | — |
| *S. dysenteriae* | $10^{-3}$ | $10^{0}$ | $10^{-6}$ | $10^{-5}$ |
| *S. paradysenteriae* | $10^{-2}$ | $10^{0}$ | $10^{-4}$ | $10^{-4}$ |
| *S. sonnei* | $10^{-1}$ | $10^{0}$ | $10^{-2}$ | $10^{-5}$ |
| *S. ambigua* | $10^{-4}$ | $10^{-4}$ | $10^{-6}$ | $10^{-5}$ |

— = no lysis. PTB = *P. pseudotuberculosis* (Spokane).

TABLE II.
Lysis After Transfer of *P. pestis* Phage on Pasteurellae and Shigellae.

| | | Agar plate cultures of | | |
|---|---|---|---|---|
| Phage transferred on | Tested on *P. pestis* | 9 PTB strains not lysed by original phage | 31 PTB strains lysed by original phage | Shigellae* |
| *P. pestis* | ++ | — | ++ | + |
| PTB (Spokane) | ++ | ++ | ++ | + |
| Shigellae* | ++ | — | ++ | ++ |
| PTB—then *S. sonnei, paradysenteriae,* or *ambigua* | ++ | ++ | ++ | ++ |
| PTB then *S. dysenteriae* | ++ | — | ++ | ++ |
| PTB then *S. dysenteriae* then PTB again | ++ | ++ | ++ | ++ |

++ = lysed by phage in dilution of $10^{-3}$ or higher.
+ = lysed by phage in dilution lower than $10^{-3}$.
— = not lysed.
PTB = *P. pseudotuberculosis.*
* Includes *S. dysenteriae, S. paradysenteriae, S. sonnei,* and *S. ambigua.*

different organisms in altering the characteristics of the original phage. The plan and the results are summarized in Table II.

It was again noted that phage first adapted to *P. pseudotuberculosis* (Spokane) and then transferred on *S. dysenteriae* lost its ability to lyse *P. pseudotuberculosis* (Spokane). This adapted phage was then tested against all 40 strains of *P. pseudotuberculosis.* It failed to lyse any of the nine strains which were resistant to the original phage, but lysed all 31 strains sensitive to the original phage. Hence this phage adapted to the Spokane strain and then to *S. dysenteriae* behaved just like the parent *P. pestis* phage in its action on *P. pseudotuberculosis* cultures.

Transfer of phage adapted to the Spokane strain on shigellae other than *S. dysenteriae* did not change its ability to lyse all the *P. pseudotuberculosis* strains. Therefore the loss of activity against certain *P. pseudotuberculosis* cultures was produced only by *S. dysenteriae.* The potency of this phage for all strains of *P. pseudotuberculosis* was completely restored by again transferring it on the Spokane culture after passage on *S. dysenteriae.* Furthermore, the original phage adapted first to shigellae, including *S. dysenteriae,* and then to *P. pseudotuberculosis* (Spokane) lysed both the shigellae and all strains of *P. pseudotuberculosis.* Therefore, it seemed that transfer of phage adapted to *P. pseudotuberculosis* on *S. dysenteriae* produced a selective blocking action resulting in loss of ability to lyse a group of cultures previously sensitive.

Tests were made to determine whether this blocking action might be due to metabolic or disintegration products of *S. dysenteriae* which, of course, would be present in phage adapted to this organism. A lysate of *S. dysenteriae* was prepared from a broth culture by adding phage adapted first to *P. pseudotuberculosis* and then to *S. dysenteriae*. This lysate was mixed with equal parts of phage adapted to *P. pseudotuberculosis* and allowed to stand at 37°C for two hours. The mixture was then added to broth and agar cultures of *P. pseudotuberculosis* using the same technic as usual. There was complete lysis in all tests. Hence, the presence of a lysate of *S. dysenteriae* did not inhibit lysis of *P. pseudotuberculosis* by phage adapted to the latter.

The plaques produced by the original *P. pestis* phage on agar cultures of *P. pestis* were uniform in size and measured about 3 mm in diameter after 24 hours. On plates of *P. pseudotuberculosis* and of shigellae, the plaques formed by the original phage varied in diameter from 0.5 to 3 mm. Large and small plaques were isolated repeatedly in attempts to separate two strains or mutants of phage, but without success. Isolated plaques gave rise to both large and small plaques even after many subcultures. Successive transfer on four species of shigellae followed by passage on *P. pseudotuberculosis* did not alter the size of plaques. The plaque size for a given culture was no different with the various adapted phages than with the original phage.

No explanation can be given at present for the alteration of phage adapted to *P. pseudotuberculosis* by subsequent transfer on *S. dysenteriae* so that it longer lyses certain strains of pasteurellae. It seemed possible that the original phage might contain two components and that the one acting on these strains could not multiply in the presence of *S. dysenteriae*. However, this is unlikely because the activity toward *P. pseudotuberculosis* was readily restored by readaptation to the Spokane strain after many transfers on *S. dysenteriae*.

The variation in plaque size shown by the original phage suggests that mutant types might be present which might vary in their host range, although attempts to separate such mutants were unsuccessful. Delbruck[2] has reported the occurrence of biochemical mutants of phage with differing requirements for cofactors for adsorption of the phage on the host bacteria. One of his mutants required tryptophan or its analogues and its adsorption was inhibited by indole. These substances are not concerned here for the medium used is rich in tryptophan and neither *P. pseudotuberculosis* nor *S. dysenteriae* produce indole. Lysis was not inhibited by the addition of lysates of *S. dysenteriae* to mixtures of *P. pseudotuberculosis* and its adapted phage.

Likewise there is no apparent explanation for the difference in behavior of those strains of *P. pseudotuberculosis* resembling *P. pestis* in their sensitivity to the phages and of those not resembling *P. pestis*. No correlation could be demonstrated between the sensitivity of the strains to the various phages and their microscopic morphology or their antigenic structure. The only detectable difference was that the majority of the strains behaving like *P. pestis* produced rough colonies while those of the other group were usually smooth.

*Summary*. Bacteriophage, originally recovered as a lytic agent for *P. pestis*, also lysed 31 of 40 strains of *P. pseudotuberculosis*. After adaptation to one of these (Spokane strain), it also lysed the remaining nine strains. When the adapted phage was transferred on *S. dysenteriae*, it reverted to its original activity; *i.e.*, it again failed to lyse the nine strains of *P. pseudotuberculosis* which resisted the original phage. Activity for all strains was restored by transferring it on the Spokane strain. Lysates of *S. dysenteriae*, mixed with the adapted phage, caused no reversion to the original selective activity for only 31 strains of *P. pseudotuberculosis*. Transfers of the adapted phage on *S. paradysenteriae, S. sonnei, S. ambigua,* or on all of these species in succession, did not alter its ability to lyse all strains of *P. pseudotuberculosis*. The size of plaques did not change during transfers on either the shigellae or the pasteurellae used.

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### 16698

# Fate and Distribution of Penicillin in the Body. II. Duration of Blood Concentration and Chemotherapeutic Effectiveness.*

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The original concept that the continuous presence of penicillin in the blood is essential for therapeutic effectiveness has been substantially weakened since the clinical reports of Tillett, McCormack, and Cambier.[1,2] These authors obtained excellent results in the treatment of lobar pneumonia on a regimen which gave detectable blood levels only intermittently. These findings were confirmed by the experimental studies of Zubrod,[3] White, Baker and Jackson[4] and Gibson.[5] The implications of such observations were discussed by Marshall[6] and Eagle.[7]

Our studies[8] on the distribution of penicillin in the organism required an analysis of the relationship between duration of therapeutic effectiveness and "blood level" as determined by *in vitro* methods.

*Material and Methods.* Albino rats of 60 to 220 g body weight were injected intramuscularly with a repository preparation consisting of a suspension of crystalline potassium penicillin G in peanut oil containing 1 mg/cc epinephrine.[9] At various intervals after injection the animals were etherized and bled from the subaxillary artery for the determination of the penicillin blood concentration by the method in current use.[10] At corresponding intervals other groups were infected. One cc of an 18 hour serum-broth culture of Streptococcus strain 4 (Group A, Type 3) and Pneumococcus Type 1 strain 6301 was inoculated intraperitoneally into the rats previously treated with the penicillin preparation. The inocula varied from $10^{-5}$ to $10^{-7}$ for the pneumococcus and from $10^{-4}$ to $10^{-6}$ for the streptococcus, corresponding to 100 - 1000 LD. Groups of 10 to 20 rats were used for control and for each alteration in the interval, or dosage, in any single experiment. Most of the untreated control animals died within 2 to 3 days. The rats which survived a three-week period were adjudged cured; occasionally the effect of treatment was verified by heart cultures of the animals sacrificed at the end of the observation period.

*Results.* (*a*) *Blood levels.* In our initial attempts to establish the duration of the penicillin levels, we encountered a number of

TABLE I.
Duration of Detectable Penicillin Levels in Rats Treated with Penicillin in Oil + 1 mg/cc Epinephrine.
Injection: 0.1 cc/100 g, intramuscular.
Rats of 80-150 g weight.

| Dose/100 g | Hr duration in<br>50% rats | <br>90% rats |
|---|---|---|
| 5 × 1000 | 13 | 14 |
| 10 × 1000 | 14 | 16 |
| 20 × 1000 | 16 | 18 |

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* Part of this material was presented at the meeting of the American Societies of Experimental Biology, Atlantic City, May, 1948.

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TABLE II.
Protection of Rats Against Pneumococcus Type I Infection at Various Intervals After Repository Penicillin Treatment.

| | | | Survivors after | | | |
|---|---|---|---|---|---|---|
| | | | 6 days | | 21 days | |
| Dose u/100 g‡ | Hr after treatment | Blood negative for hr | Survivors infected | % survivors | Survivors infected | % survivors |
| Controls | — | — | 26/134 | 19.4 | 14/134 | 10.4 |
| 10,000 | 14 | * | 8/ 10 | 80 | 8/ 10 | 80 |
| | 17 | 1 | 11/ 20 | 55 | 6/ 20 | 30 |
| | 20 | 4 | 8/ 20 | 40 | 4/ 20 | 20 |
| | 22 | 6 | 7/ 10 | 70 | 6/ 10 | 60 |
| | 24 | 8 | 14/ 25 | 56 | 7/ 25 | 28 |
| | 28 | 12 | 2/ 10 | 20 | 2/ 10 | 20 |
| 20,000 | 16 | * | 7/ 10 | 70 | 6/ 10 | 60 |
| | 18 | † | 20/ 30 | 66.6 | 16/ 30 | 53 |
| | 20 | 2 | 31/ 50 | 62 | 21/ 50 | 42 |
| | 25 | 7 | 6/ 13 | 46 | 2/ 13 | 15.4 |
| | 35 | 17 | 4/ 13 | 31 | 1/ 13 | 7.7 |

* 50% of rats negative.
† 90% ” ” ”
‡ in 0.1 cc/100 g.

discrepancies. From experiments conducted on a large number of rats, it became apparent that the absolute weight of the animal —using the same dose/weight—influences the duration of blood levels. The larger animals (over 150 g) gave longer durations due to the greater absolute dose injected. In order to minimize variables, only experiments conducted on rats of 80-150 g weight are considered in this paper.

The duration of blood level is expressed in the most significant manner by indicating the end period of detectable penicillin in 50% and 90% of the animals. (Table I). The establishment of this effect in 100% of the animals is exposed to greater error, as is the case in other dose-response relationships.

(b) *Pneumococcus Type I Infection.* From the experiments presented in Table II, *it is evident that the therapeutic activity outlasts the duration of the detectable blood level.* For instance, rats infected 20 hours after treatment had no penicillin in their blood for the last 2 hours before infection, yet in 62% death was significantly delayed and 42% were cured. In general, a definite protective effect was given by the repository treatment 6-8 hours after the end of the penicillin blood level.

(c) *Hemolytic Streptococcus Infection.* Four experiments were carried out on a total of 250 rats. The same bacterial strain was used for *in vitro* as for *in vivo* experiments. The results are not as clear-cut as in the pneumococcal infection, due probably to the heavier inocula used in order to compensate for the less *regular* susceptibility of rats to streptococcal infection. However, in this infection also, chemotherapeutic effectiveness appears to be present for relatively long periods (4-8 hours) following non-detectable blood levels. In one experiment, for instance, out of 30 rats infected 2 to 6 hours after the disappearance of penicillin from the blood, 10 rats survived an observation period of 21 days. Seventy-five percent of a group of 20 controls died within 2 days; the entire group within 8 days.

*Conclusion.* It may be concluded that our present bacteriological methods cannot give the value of the *minimal* penicillin concentrations required for therapeutic activity. It is certain that this concentration in the heavy bacterial infection used is below 0.015–0.03 u/cc serum, which is the sensitivity of the bacteriological method.

Our present finding explains only in part the well-documented opinion of other authors that it is not necessary to maintain continuous penicillin blood levels for the whole period of treatment. A more important factor responsible for this is probably the *additive chemo-*

*therapeutic effect* of repeated doses. This effect is obvious from the experiments reported in the literature indicating that *fractions* of the curative dose, repeated at such intervals as to leave the organism without penicillin for the greatest part of the treatment period, result in cure. This is the case in spirochetal[11] as well as in bacterial[3] infections. The *additive effect* might depend on biological changes induced by sub-curative doses on the micro-organism, on reduction of the bacterial population, and on the immunological response of the host. The experiments of Kelly and Schnitzer[12] would suggest furthermore that the immunological response might influence the susceptibility of the micro-organism to chemotherapy.

The immunological response itself can be influenced by the treatment schedule, according to the recent observations of Kilbourne and Loge.[13] In hemolytic streptococcal pharyngitis, the antistreptolysin titer is higher with *intermittent* penicillin injections than it is with continuously sustained blood levels.

Another phenomenon which accounts for the independence of therapeutic effect duration from blood level is revealed in the experiments of Grunberg, Schnitzer, and Unger.[14] Penicillinase injected *after* the total disappearance of penicillin from the host tissue still blocks the therapeutic effect in the local streptococcal infection of mice. Thus, the presence of the penicillin in the micro-organism is more prolonged than in the host.

In our own experiments, part of the therapeutic effect existing beyond the blood level is due to penicillin accumulated in the tissues. How much of this prolonged therapeutic effect is dependent upon the undetectable amounts present in the blood, in comparison with the penicillin accumulated in the organs and tissue fluids, remains an open question.

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## 16699 P

# 4-Amino Pteroylglutamic Acid (Aminopterin), Pteroylglutamic (Folic) Acid, and Response of Frogs, *Rana clamitans*, to Estrogens.*

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It has been shown that chicks and monkeys (*Macaccus rhesus*) maintained on a diet deficient in pteroylglutamic (folic) acid exhibit a decreased response to estrogenic substances.[1-3] It has been further demonstrated by Franklin, Stokstad, and Jukes[4] that folic acid deficiency in mice may be accelerated by a chemical antagonist of folic acid ("crude antagonist", Lederle). Hertz,[5] using the same antagonist, has shown that it interferes with the estrogen response of the chick genital

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* This investigation was supported by a research grant from the National Cancer Institute of the National Institute of Health, U. S. Public Health Service.

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TABLE I.
Effects of Various Levels of 4-Aminopteroylglutamic Acid, Pteroylglutamic Acid, and Estradiol Benzoate on the Growth of Oviducts of *Rana clamitans*.

| Series No. | No. of frogs | Aminopterin per inj., mg | Folic acid per inj., mg | No. of inj. per wk | Estradiol per wk, mg | Oviduct response of female frogs | |
|---|---|---|---|---|---|---|---|
| | | | | | | After 2 wks of estradiol | After 3 wks of estradiol |
| I* | 9 | .05 | 0 | 3 | .1 | + | + |
| ” | 10 | .10 | 0 | 3 | .1 | + | + |
| ” | 5 | 0 | 2.0 | 3 | .1 | +++ | ++++ |
| ” | 5 | .10 | 1.0 | 3 | .1 | + | + |
| ” | 10† | 0 | 0 | 0 | .1 | +++ | ++++ |
| ” | 5 | 0 | 0 | 0 | 0 | — | — |
| II* | 5 | .05 | 0 | 3 | .1 | + | |
| ” | 13 | .10 | 0 | 3 | .1 | + | |
| ” | 11 | .20 | 0 | 3 | .1 | + | |
| ” | 2 | 1.0 | 0 | 2 | .1 | + | |
| ” | 5 | 0 | 1.0 | 3 | .1 | ++++ | |
| ” | 5 | 0 | 2.0 | 3 | .1 | ++++ | |
| ” | 5 | .05 | 1.0‡ | 3 | .1 | + | |
| ” | 4 | .1 | 2.5‡ | 3 | .1 | + | |
| ” | 5 | .25 | 2.5‡ | 3 | .1 | + | |
| ” | 5 | 0 | 0 | 0 | 0 | — | |
| ” | 5 | 0 | 0 | 0 | .1 | +++ | |

* Series I animals were simultaneously treated with estradiol and with substance(s) listed. Series II animals were pretreated for 2-3 weeks with the drug(s) listed and then in addition were injected with estradiol. (Administration of the drug(s) aminopterin, folic acid, or both were continued throughout estradiol treatment.)

† 5 animals of this group also received 0.4 cc of distilled water 3 times per week.

‡ Folic acid level was raised after 2 weeks treatment to 5 mg per injection.

| | |
|---|---|
| + | Slight oviduct enlargement, no coiling. |
| +++ | Oviducts enlarged and coiled. |
| ++++ | Marked oviduct enlargement and coiling. |
| — | Oviducts small, thin, and uncoiled. |

tract. The 4-amino analogue of pteroylglutamic acid was described as being strongly antagonistic to folic acid in the *Streptococcus fecalis* R test.[6] Goldsmith, Tobias, and Harnly[7] reported that both the "crude antagonist" and 4-amino pteroylglutamic acid (aminopterin) prevented the development of *Drosophila melanogaster* larvae into adults. This inhibition by the "crude antagonist" could be overcome in a number of cases by the addition of folic acid to the diet. However, thus far, the inhibitory effect of aminopterin on the development of Drosophila larvae has not been counteracted or reversed by several levels of folic acid. Franklin, Stokstad, and Jukes[8] found that mice died within a few days after being placed on a diet containing 1 mg or more of aminopterin per kilo of diet. At a level of 0.3 mg per kilo the aminopterin depressed the blood values. This could be reversed by high dosages of folic acid. However, large amounts of folic acid did not overcome the effect resulting from higher concentrations of aminopterin. Recently, Hertz described the decreased response of the chick[9] and the rat[10] to estrogen following treatment with aminopterin. High levels of folic acid reversed this effect.[10]

In the light of the above data, and since it had been established previously that estradiol injections in newly metamorphosed frogs are followed by a pronounced oviduct response within 2 to 3 weeks,[11] it was considered worthwhile to ascertain the action of aminopterin and its effect on the estrogen response in frogs.

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*Experimental.* This paper presents preliminary data on a group of about 100 newly metamorphosed frogs (*Rana clamitans*) treated parenterally with various dosage levels of aminopterin, folic acid, and estradiol benzoate. The course of treatment and levels of drugs administered are outlined in Table I. The frogs were divided into two series. In series I, the frogs were treated with aminopterin† or folic acid,† or both, and were simultaneously treated with estradiol benzoate.‡ In series II, frogs were pretreated with aminopterin alone, folic acid alone, or both for a period of 2 to 3 weeks. At this time, estradiol was added to this regimen. The volume of fluid injected was controlled as closely as possible, and no frog received more than 0.4 cc of fluid per injection. In addition, the site of puncture was carefully held closed to prevent any leakage of injected fluid.

The data indicate that treatment with aminopterin decreased the response of the female frog oviducts to estradiol. Oviducts of the estradiol treated females showed marked enlargement and coiling. However, the oviducts of the females which received estradiol and aminopterin simultaneously, or which were pretreated with aminopterin and then were given estradiol plus aminopterin, showed very slight enlargement and no coiling.

Simultaneous administration of folic acid and estradiol was followed by oviduct growth and coiling in the females equal to, but not greater than that observed in the females treated with estradiol alone. However, pretreatment with folic acid for 2 to 3 weeks followed by injections of estradiol plus folic acid resulted in oviduct growth which was greater than that observed in the females which received only estradiol.

In 4 groups of frogs, in addition to aminopterin and estradiol, supplements of folic acid were administered in ratios of 10, 50 and 100 to 1 of folic acid to aminopterin. In none of these female frogs were the oviducts grossly different from those in the females treated only with aminopterin and estradiol.

The data also indicate that those animals treated with aminopterin appeared to show a higher mortality rate than those receiving supplements of folic acid in addition to aminopterin. The majority of aminopterin treated frogs which died, did so 3 to 4 weeks following initiation of treatment with aminopterin, irrespective of level of drug administered.

Histological studies of the oviducts and other organs such as liver, spleen, and adrenal will be reported at a later date.

*Summary.* 4-amino pteroylglutamic acid (aminopterin) markedly decreased, whereas, folic acid increased the response of the oviducts of newly metamorphosed female frogs (*Rana clamitans*) to estradiol. The aminopterin effect could not be reversed by folic acid in ratios as high as 100:1 of folic acid to aminopterin.

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† The 4-amino pteroylglutamic acid was generously supplied by the late Dr. Y. SubbaRow, and the pteroylglutamic acid by Dr. A. L. Franklin of Lederle Laboratories.

‡ The estradiol benzoate was generously supplied by Dr. F. F. Yonkman and Dr. F. L. Mohr of Ciba Pharmaceutical Products.

## 16700 P

# A New Method for the Electrical Recording of Mechanical Deformations.

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Pressure changes or spacial displacements may be recorded by a number of electrical devices,[1-10] based on changes in capacity, resistance or the piezo electric effects. Variations in potential are amplified and recorded photographically. On referring to these methods Green[11] states that "while many of them are admirable in concept, their complexity and cost have so far prevented their wide acceptance".

The system about to be described is of low cost, easy to make and of almost universal application. It depends on the variations in resistance occurring in a glass capillary (c) containing equal volumes of alcohol and glycerine; copper wires are inserted into the two ends one of which is fixed ($e_2$) and the other ($e_1$) which is movable, being connected to a membrane (m) or any other mechanical device which allows for displacement in space. The fixed electrode ($e_2$) can be moved in relation to the movable one ($e_1$) by means of a screw. The movable electrode should be of small diameter (approximately 0.75 mm) so its mass will have no influence on the experiment. The ends of the electrodes are connected to points a and b of the electric system represented in Fig. 2, consisting of a battery of approximately 10 volts and a switch $I_1$, which on closing sends the current through the variable resistance $R_1$. The proper voltage is obtained by altering the position of the cursor in this resistance. The

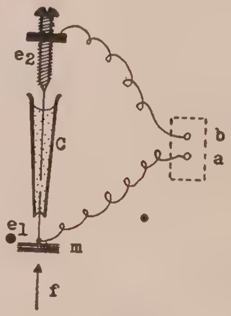

FIG. 1.
A fixed electrode ($e_2$) and a movable one ($e_1$) are inserted into the capillary tube C, which contains the electrolyte (alcohol and glycerine). The electrode $e_1$ is joined to the membrane (m), which is displaced by the force (f). Terminals a and b are connected to points similar to those shown in Fig. 2.

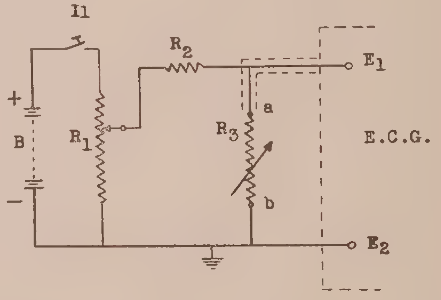

FIG. 2.
The electrical circuit consists of a battery B, a switch $I_1$, and resistances $R_1$ and $R_2$, each 250,000 ohms. Variable resistance $R_3$ corresponds to the capillary system in Fig. 1. The union between a and $E_1$ is shielded. $E_1$ and $E_2$ are entrances to the electrocardiograph (E.C.G.).

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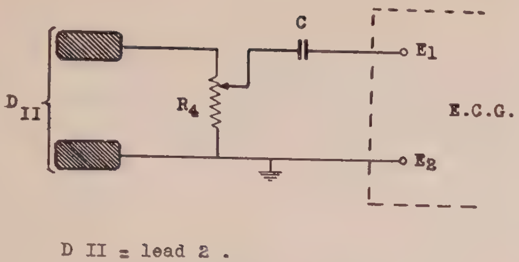

FIG. 3.
Additional circuit for recording of R wave of E.C.G. $D_{11}$ = lead 2. $R_4$ = 250,000 ohm variable resistance. C = 0.2 u F condenser.

potential differences produced at the extremes of $R_3$ (the capillary's electrolytic resistance) may be amplified through a clinical electrocardiograph (E.C.G.) or another amplifying device, when the movable electrode is displaced.

It has been possible to make an immediate and accurate record of a whole range of mechanical manifestations in the circulation system by means of this apparatus. Suitable capsules are used in each case as fully described elsewhere.

In order to relate the mechanical activities recorded in this way with other manifestations of the heart's activity we have superimposed on the mechanical tracing the R wave of the electrocardiogram by using an additional circuit with a variable resistance and a condenser (Fig. 3).

The different tracings obtained in man are recorded in Fig. 4: carotid pulse (I), jugular pulse (II), venous pulse together with the R wave of the electrocardiogram (III), radial pulse and R wave (IV), and finally the apex beat together with the R wave of the electrocardiogram (V).

Further applications of this method will be described later.

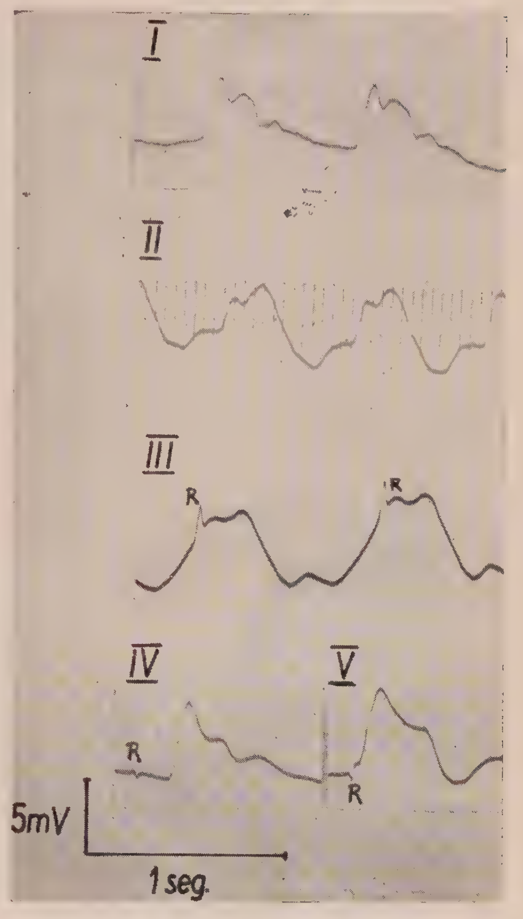

FIG. 4.
Tracings obtained in men: (I) Carotid pulse. (II) Venous pulse (jugular vein). (III) Venous pulse and R wave of the E.C.G. (IV) Radial pulse and R wave. (V) Apex-beat and R wave.

*Summary.* A new electrical method for recording mechanical variations is described which uses the differences in potential obtained by changes in the electrolytic resistance of a glass capillary filled with alcohol and glycerine. The method is specially useful in the study of mechanical changes in the cardio-vascular system.

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## 16701

# Influence of Vaccine Virus on a Transmissible Leukemia of Mice.*

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The relation of extraneous viruses to neoplasms has been the subject of comparatively few investigations, scattered over the past 25 years. Levaditi and his associates[1,2] found that certain pox viruses proliferate in epitheliomas of rodents and sometimes cause necrosis and impairment of growth of the tumors. Rivers and Pearce[3,4] studied the influence of Virus III on a rabbit carcinoma, but could find no appreciable alteration of the degree of malignancy.

More recently, it has been demonstrated that vaccine virus will proliferate in mouse sarcoma 180, and furthermore, that transplants of fragments of infected tumor grow much more slowly and less successfully than controls.[5] The explanation was offered that this effect was produced by a direct action of the virus on the neoplastic cells, perhaps by a kind of competitive biochemical antagonism. The study has now been extended to leukemias of mice. These neoplasms provide an exceptionally satisfactory test material, both in their close resemblance to the corresponding human disease and in their readily predictable and high grade of malignancy.

Mice of a closely inbred line of the Ak strain and a leukemia designated 9417 have been employed. This tumor is lymphoid in type and at post-mortem the dominant lesions are usually found to be in liver, spleen, and mesenteric nodes, all of which may be greatly enlarged.

The strain of vaccine virus used was adapted to mouse brain more than a year ago, and has been carried along since by frequent intracerebral passage.[5] It is lethal when inoculated directly into the brain, but moderate doses may be given subcutaneously or intravenously without the development of any gross lesions or impairment of health. The virus was given as a suspension of infected mouse brain in 5 parts of plain broth containing 1,000 units per cc of both penicillin and streptomycin and 0.5 mgm heparin.

Leukemia 9417 is readily transmitted within this strain of mice, becoming established in, and killing approximately 100% of the hosts within a few weeks.[6] For transmission, the enlarged spleen of an animal dying of the disease was macerated in 10 cc of normal saline, and 0.1 cc was injected intraperitoneally.

In a first experiment leukemic cells were implanted in 50 young mice. Of these, 23 were kept as controls and received no further treatment. The remaining 27 were given vaccinia virus parenterally after periods of time sufficiently long to allow no reasonable doubt that the leukemia had meanwhile become well-established. One group of 17 received on the seventh day after the leukemic transplant 0.2 cc of vaccine virus suspension intravenously. A second group of 10 mice were given, on the fifteenth day, 0.2 cc of virus suspension intravenously and at the same time 0.5 cc intraperitoneally. The rates at which the control and the treated

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* This investigation was aided by grants from the Jane Coffin Childs Memorial Fund for Medical Research, the Holmes Foundation, Inc., and the National Cancer Institute of the National Institute of Health, U. S. Public Health Service.

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TABLE I.
Number of Mice Surviving After Inoculation of Leukemic Cells.

| Day: | 13 | 16 | 19 | 22 | 25 | 54 |
|---|---|---|---|---|---|---|
| Controls | 23/23 | 3/23 | 0 | — | — | — |
| Treated with virus on Day 7 | 17/17 | 7/17 | 6/17 | 4/17 | 2/17 | 2/17 |
| Treated with virus on Day 15 | 10/10 | 7/10 | 6/10 | 4/10 | 2/10 | 2/10 |

TABLE II.
Number of Mice Surviving After Inoculation of Leukemic Cells.

| Day: | 13 | 16 | 19 | 22 | 25 | 28 | 35 |
|---|---|---|---|---|---|---|---|
| Controls | 19/20 | 16/20 | 9/20 | 4/20 | 2/20 | 1/20 | 1/20 |
| Treated with virus on Day 7 | 13/15 | 7/15 | 5/15 | 4/15 | 4/15 | 4/15 | 4/15 |
| Treated with virus on Day 12 | 15/15 | 12/15 | 7/15 | 4/15 | 2/15 | 2/15 | 2/15 |

animals died may be seen in Table I.

It may be noted that all of the control mice were dead on the 19th day after inoculation of leukemic cells. In contrast, deaths occurred less rapidly in both groups receiving virus, and no less than 4 animals (15%) were still alive at least until the 54th day after transplantation of leukemia. Two have survived 194 days.

The experiment was repeated with the modification that the group of animals treated late in the disease received the injection of virus on the 12th day instead of on the 15th day. There were 20 control mice and 15 mice in each of the groups receiving virus. Table II records the findings.

It is apparent that the leukemia is now somewhat less acute than before, yet among the controls the mortality at the end of the 4th week is nearly 100%. A difference between the groups is first evident at that time when, with all but one of the control animals dead there are still 6 (20%) of the 30 treated mice alive. Furthermore, 4 of these have now survived at least until the 170th day after transplantation of the leukemia.

All of the mice that died, both treated and untreated, showed gross evidence of leukemia, with enlargement of liver, spleen, or lymph nodes. Portions of the organs removed from animals that had received virus were examined for vaccinia by intradermal injection of rabbits and by inoculation of chorioallantoic membranes. Virus was demonstrable in about 25%. Whether this represents a fair estimate of the presence of virus is not clear, since it may well be that antibody was circulating in sufficient concentration to interfere with the tests.

It would appear that the introduction of vaccinia virus by parenteral injection in the course of mouse leukemia may result in the prolongation of life of a significant number of hosts. Whether the animals still alive will ultimately die of leukemia remains to be seen. It could be supposed that they will, and that the delay in the time of their death is merely a consequence of undernutrition brought on by systemic vaccinial infection; but no evidence of appreciable complicating general injury to the hosts has yet been uncovered. The weights of all groups of animals were taken several times a week, and none failed to gain.

It is hardly possible to compare the prolongation of life of leukemic mice brought about by orthodox chemotherapeutic or physical agents with the effect observed as a result of the injection of virus. In one case it is customary to employ repeated doses of the material on trial and to begin very soon after the leukemia has been transplanted into the subject. Here, on the other hand, a dose of virus has been given only on a single day and that only relatively late in the course of the disease.

The mechanism of the observed effect is not clear. It is reasonable to suppose that a direct action of virus on neoplastic cells takes place, but the reaction may then be modified by the operation of immune bodies.

*Summary.* In 2 experiments, 100 mice of the inbred Ak strain were inoculated with leukemia 9417. One week or two weeks later

57 animals received parenteral injections of living vaccinia virus. The 43 controls received no treatment.

Of the controls all but one (98%) were dead within 4 weeks after the leukemic transplant. The lives of at least 10 (18%) of the mice receiving virus seemed to be significantly prolonged, and 6 of these have survived more than 5 months.

The authors wish to express their appreciation for the generosity of Dr. Joseph Burchenal of the Sloan-Kettering Institute, who supplied the tumors, as well as most of the mice employed.

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### 16702

## Search for the Brown-Pearce Tumor XYZ Factor in Rabbit Spleen.

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Some years ago it was reported that frozen and preserved Brown-Pearce tumor tissue contained a factor called, for lack of a better term, the XYZ factor, which, when injected 2 weeks before the Brown-Pearce tumor transplantation resulted in increased incidence of, and larger metastases, and a shorter survival period after transplantation.[1-4] The factor was different from the Duran-Reynals spreading factor (hyaluronidase),[5-8] nor was the factor present in normal rabbit testicle,[5,6] in the tissue of an adenocarcinoma of the rabbit uterus (Greene),[9] in Bashford Carcinoma 63,[10] nor in Sarcoma 180 of the mouse.[10] A review of the literature revealed that Haaland[11] and Leitch[12] had described a similar factor for Bashford Carcinoma 63 and it was possible to confirm their work.[13] The Bashford Carcinoma 63 XYZ factor seemed to be specific in that the growth of this tumor was not affected by similarly prepared material from mouse Carcinoma 48, mouse Sarcoma 37, nor by the XYZ factor from the Brown-Pearce tumor.[14,15] Much has been written concerning the presence of inhibiting and enhancing materials said to be present in the spleen.[16] In resuming work on the Brown-Pearce XYZ factor after a lapse of some 10 years, preliminary experiments were performed in an attempt to recover the factor from the spleens of normal rabbits and of rabbits 2 weeks after the injection of the factor.

*Materials and methods.* In the 2 experiments 52 rabbits were employed. Each rabbit was about 3 months of age, except for

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[12] Leitch, Archibald, *The Lancet*, 1910, **88**, 991.

[13] Casey, Albert E., Proc. Soc. Exp. Biol. and Med., 1933, **30**, 674.

[14] Casey, Albert E., Proc. Soc. Exp. Biol. and Med., 1933, **30**, 1025.

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TABLE I.
Course of Brown-Pearce Tumor in Rabbits Transplanted Subcutaneously.

| | Rabbits, No. | Tumor takes | Tumor per animal inoculated | | | Per animal with tumor |
|---|---|---|---|---|---|---|
| | | | Met. foci, No. | Vol. met., cc | Total tumor, cc | Met. foci, No. |
| Spleen (XYZ) | 12 | 3 (25%) | 0.3 | 2.1 | 11.7 | 1.3 |
| Spleen (normal) | 12 | 4 (33%) | 1.8 | 10.7 | 18.5 | 5.5 |
| Controls | 13 | 3 (23%) | 1.7 | 19.3 | 33.4 | 7.3 |
| XYZ (BP tumor) | 3 | 2 (67%) | 10.7 | 45.0 | 112.7 | 16.0 |
| 8 Prior Experiments with the Subcutaneous Inoculation of BP Tumor. | | | | | | |
| Controls | 52 | 16 (31%) | 2.7 | 12.8 | 21.6 | 4.4 |
| XYZ (BP tumor) | 58 | 44 (76%) | 7.7 | 31.1 | 46.7 | 10.2 |

7 adult males used in obtaining the transfer material and in the preparation of the spleens. The young animals were divided into matched groups of the same breed and all were obtained from the same local breeder.

The spleens were prepared as follows: Two normal rabbits were killed by air injection and the spleen removed aseptically, placed in a sterile tube and stored in the deep freeze chamber at 0°F. The preserved spleen after 14 days in the first and 68 days in the second experiment, was removed from the deep freeze, emulsified in normal saline (1-3 dilution) and 0.3 cc of the emulsion injected subcutaneously into 5 rabbits in the first experiment and into 8 rabbits in the second experiment.

For XYZ material a rabbit bearing the Brown-Pearce tumor was killed by air injection 13 days after intratesticular transplantation. The large testicular tumor was removed aseptically, 1.5 cc used immediately for transfer (A) and 6 cc placed into sterile tubes without glycerin or other preservative and stored at 0°F (B). The transfer tissue (A) was emulsified in 4 cc of normal saline and 0.2 cc of the emulsion injected subcutaneously or intratesticularly into 15 rabbits. The transplantation was successful indicating that the tumor used was viable. One portion of the frozen tumor tissue (B) was removed 7 days later, 3 cc emulsified in 10 cc of normal saline and 0.33 cc of the emulsion was injected subcutaneously into the back of 2 rabbits which were killed by air injection 16 days later, the spleens removed aseptically, placed in sterile tubes, and stored in the deep freeze chamber at 0°F (C). The spleens thus obtained (C) were removed from the deep freeze after 14 days in the first and 68 days in the second experiment, emulsified in normal saline (1-3 dilution) and 0.3 cc of the emulsion injected subcutaneously into 5 rabbits in the first and into 8 rabbits in the second experiment. In the first experiment a second batch of frozen Brown-Pearce tumor tissue (XYZ material, marked B) was removed after 37 days in the deep freeze, emulsified in normal saline (1-3 dilution) and 0.3 cc injected subcutaneously into 5 rabbits. The material used was sterile when cultured in thioglycollate medium, and microscopic sections of biopsies of the frozen and fresh spleen and tumor were made and examined at each stage of the experiment.

In the first experiment, 8 days after injection of the spleen emulsions and the Brown-Pearce XYZ material, each animal in the 3 groups (with spleen from XYZ animal, with spleen from normal animal, with Brown-Pearce XYZ material; one animal had died meanwhile of intercurrent disease) together with the previously matched but uninoculated controls were injected subcutaneously into the back in numerical order with an emulsion of Brown-Pearce tumor tissue in normal saline.

In the second experiment there were 3 groups of 8 animals in each. The first group was injected with an emulsion of spleen preserved 68 days at 0°F from the animal which had received Brown-Pearce XYZ (as described above); the second group was injected with an emulsion of spleen taken from a

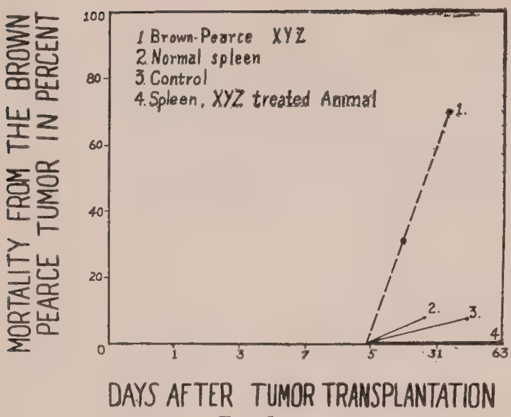

FIG. 1.

normal rabbit and preserved for 68 days at 0°F; the third group were control animals. Twenty days after the injection of the splenic emulsions into the 2 experimental groups the animals of these 2 and those of the control group (24 animals in all) were injected subcutaneously with 0.2 cc of an emulsion (1-3 dilution) of Brown-Pearce tumor tissue in numerical order. In addition a stock animal (NZW) was injected intratesticularly with 0.2 cc of the emulsion. Biopsy showed that this tumor was nearly all living, the animal having been inoculated intratesticularly.

*Results.* The results are summarized in Fig. 1 and in Table I. One animal from the controls and one from the spleen XYZ series, another from the Brown-Pearce XYZ group and one from the normal spleen group died of intercurrent disease between 2 and 17 days after tumor transplantation. These were eliminated from the results because mortality from the tumor does not occur until 20 days or longer after inoculation.

There was no statistically significant difference in the success of transplantation, in the mortality, in the number of metastatic foci, nor in their volume, nor in the total tumor primary and metastatic per animal inoculated between the 3 groups. There was no evidence that the emulsions of the splenic tissue had in any way significantly affected the course of the Brown-Pearce tumor.

The Brown-Pearce XYZ group containing only 3 animals could not be analyzed statistically but it was consistent with previous experience on the intracutaneous inoculation of the Brown-Pearce tumor (Table I). In 8 prior experiments at the Rockefeller Institute,[3] at the Louisiana State University[10] and at the Baptist Hospitals[10] there were 52 controls and 58 B.P. XYZ animals inoculated with Brown-Pearce tumor subcutaneously into young rabbits. The incidence of tumor proven by autopsy was among all animals inoculated, 31% in the controls and 76% among the XYZ treated animals, metastatic foci 2.7 and 7.7, volumes of metastatic tumor 12.8 and 31.1 cc, total tumor 21.6 and 46.7 cc; metastatic foci among animals with tumor was 4.4 in the controls and 10.2 in the XYZ animals. The results in the XYZ group in the present experiments were in accord with this prior data.

*Discussion.* Stern and Willheim published a review of the voluminous literature on the effect of spleen extracts on transplanted tumors.[16] Much of the work reviewed was done without controls or without an adequate number of animals for statistical analysis. Woglom[17] found that fresh splenic implants increased resistance to tumor transplantation but that crushed, frozen or thawed mouse spleen had no effect upon resistance to Bashford carcinoma 63 of the mouse. Fardon, Brotzge and Loeffler[18] obtained resistance to mouse mammary carcinoma 15091 A by means of extracts of fresh (unfrozen) mouse spleen and not with heterologous spleen. Since our work was done with frozen and not with fresh spleen it is in accord with previously published work and there is no reason to suspect that the Brown-Pearce XYZ factor may be found in the spleen of normal or of XYZ injected animals.

*Summary.* The Brown-Pearce tumor XYZ factor could not be prepared from the spleen of normal rabbits nor from the spleens of rabbits previously injected with the factor. There were two experiments comprising 52 rabbits and further controlled by prior experiments involving 110 young rabbits likewise injected intra- or subcutaneously with the Brown-Pearce tumor.

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### 16703

## Influence of Alloxan Diabetes on Coagulability of the Blood.*

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The possibility that the pancreas may influence the coagulation of the blood has been considered by several investigators. Boldyreff[1,2] has reported that pancreatectomy results in a temporary delay of the clotting time and that the establishment of a pancreatic fistula causes a significant and permanent prolongation of the clotting time as well as a bleeding tendency. Recently Hecht[3] suggested a relationship between the internal secretion of the pancreas and the coagulability of the blood. He observed that diabetes was more common in families in which cases of sporadic hemophilia occurred, the criterion of sporadic hemophilia being the absence of the disease in the 4 preceding generations. This observation led Hecht to study the effect of alloxan on the clotting time in rats. He found that the clotting time of recalcified plasma was delayed in those animals which responded to alloxan and developed hyperglycemia.

In the present paper the influence of alloxan on the coagulation process was studied by means of the coagulation time and by various other methods which are more specific in detecting the basic disturbances in the coagulation mechanism.

*Experimental.* The studies were carried out on 10 adult male rabbits. The alloxan monohydrate in a 4% neutral solution in physiological saline was injected intravenously. The dose was of 200 mg per kilo of body weight. To counteract the transitory hypoglycemia resulting from the drug, 5 cc of 50% glucose in water were given every 2 hours by stomach tube for the first 10 hours.

The blood sugar level was determined every 2 hours for the first 10 hours and then every 2 days using the method of Folin and Wu[4] on venous blood obtained from an ear vein. On the second, fourth, seventh, tenth and fourteenth day samples of blood were obtained from the ear artery of the animal in a syringe coated with Silicone (General Electric Dri Film 9987) through a needle also coated with Silicone. Care was taken to avoid foaming and contamination with tissue juice. Two 1 cc samples of blood were immediately transferred to serological tubes with a uniform internal diameter of 11 mm, kept in a water bath at the constant temperature of 37°C and the clotting time determined according to a modified Lee-White technic.[5] The remaining blood was decalcified by the addition of 0.1 M sodium oxalate in the ratio of one to ten. The prothrombin consumption was determined in the serum obtained from the samples of blood used for the determination of the clotting time according to the technic of Quick.[6] The oxalated plasma, obtained by centrifuging the oxalated blood for 10 minutes at 2,000 r.p.m. was used for the determination of the clotting time of recalcified plasma, prothrombin time, relative concentration of the "labile factor", antithromboplastin and antithrombin activities. Both prothrombin time and clotting time of recalcified plasma were determined by the method of Quick.[7] The

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† Department of Internal Medicine, University of Roma, Italy. Senior Research Fellow, National Institute of Health.

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[5] Quick, A. J., Honorato, C. R., and Stefanini, M., *Blood*, 1948, **3**, 1120.

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TABLE I.
Relationship Between Blood Glucose Level, Clotting Time of Whole Blood, Prothrombin Consumption, Prothrombin Time, Clotting Time of Recalcified Plasma and Relative Concentration of the “Labile Factor” of Prothrombin, 7 Days After Intravenous Injection of Alloxan Monohydrate (200 mg per kilo body weight).

| Rabbit No. | Blood glucose level, mg per 100 cc blood | Prothrombin time, sec. | Clotting time,* min. | Clotting time of recalcified plasma, sec. | Relative conc. of “labile factor,” % | Prothrombin consumption | |
|---|---|---|---|---|---|---|---|
| | | | | | | 1 hr sec. | 2 hr |
| Control | 117.4 | 6 | 12½ | 95 | 100 | 16 | 21 |
| ” | 125.6 | 6½ | 11½ | 120 | 85 | 23 | 28 |
| ” | 106.0 | 6 | 13 | 95 | 100 | 11 | 15½ |
| ” | 112.8 | 7 | 11 | 115 | 95 | 10½ | 17 |
| 1 | 382.1 | 6½ | 15 | 120 | 100 | 13 | 21 |
| 2 | 122.4 | 6 | 12 | 135 | 90 | 12 | 18 |
| 3 | 402.7 | 7 | 17½ | 160 | 100 | 22 | 30 |
| 4 | 257.4 | 7 | 18 | 150 | 100 | 20 | 24 |
| 5 | 442.8 | 6 | 16½ | 150 | 80 | 10 | 16½ |
| 6 | 239.7 | 6½ | 20 | 165 | 100 | 14 | 19 |
| 7 | 108.3 | 6½ | 9½ | 95 | 100 | 11½ | 14 |
| 8 | 193.7 | 6 | 17½ | 120 | 95 | 17½ | 19 |
| 9 | 320.4 | 6 | 14 | 150 | 100 | 10 | 18 |
| 10 | 379.5 | 6 | 16 | 150 | 85 | 14 | 18½ |

* Arterial blood.

relative concentration of the “labile factor” and the antithrombin activity were determined according to technics recently described.[8,9]

*Results.* Two rabbits out of the 10 investigated failed to exhibit any modification of their blood sugar level. This is not surprising as occasionally animals have been found by other investigators[10] which resist the action of alloxan. The other 8 gave a typical response and the modification of their blood sugar level is presented in Table I. Two hours after injection the rabbits presented a transitory hyperglycemia followed, about 4 hours later, by a hypoglycemic phase which persisted until the eighth hour. On the second day the rabbits showed a sharp elevation of the blood sugar level, which reached its maximum level on the fourth day and then remained stationary. Correspondingly a marked glycosuria was noted.

Data showing the effect of alloxan on various constants of the clotting process are shown in Fig. 1 and Table I. The clotting time of whole blood and that of recalcified plasma appeared to be slightly delayed without any appreciable difference between the fourth and the fourteenth day of experimental hyperglycemia. The difference between these animals and normal controls is small but consistent. As observed by Hecht in rats, transitory reduction of the hyperglycemia to normal with injection of 4 units of insulin per kilo weight had no effect on the behavior of the clotting time. The prothrombin time was normal in all treated and untreated animals. The prothrombin consumption, taken one and 2 hours after the completion of clotting, showed wide variations both in normal and treated animals, without any direct relationship to the blood sugar level. Determination of the relative concentration of the “labile factor” showed no difference between treated and untreated animals.

The possible presence of an anticoagulant was ruled out. The addition of half volume of whole blood and plasma of normal and alloxan treated animals gave a clotting time close to the arithmetical average of the two. The possibility of the presence of an antithromboplastin was excluded as both normal and treated rabbit plasmas gave similar curves when their prothrombin times were determined with thromboplastin of varying

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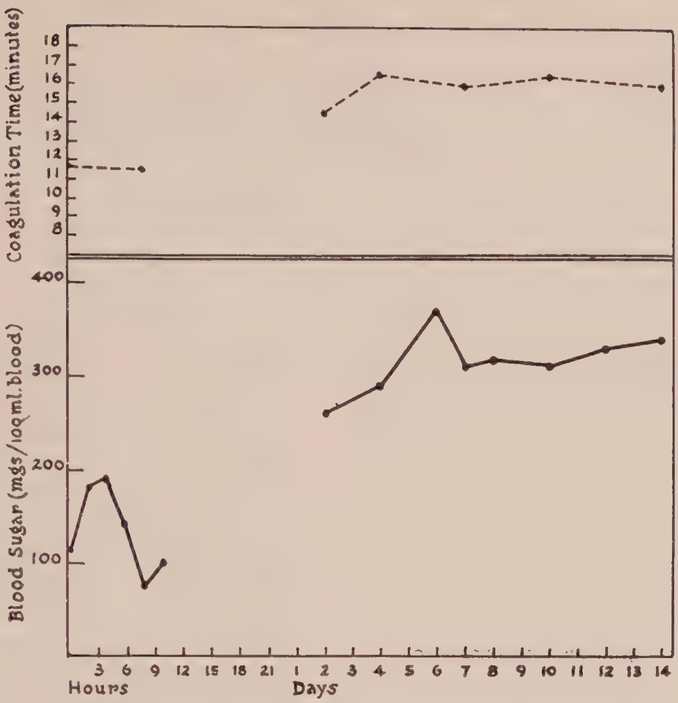

FIG. 1.

Behavior of the clotting time of whole blood and blood sugar level after the intravenous injection of alloxan monohydrate (200 mg per kilo body weight) in rabbits (average figures).

strength prepared by serial dilution in physiological saline. Normal and pathological plasmas also showed a similar antithrombin activity, when tested immediately or after incubation.[9]

*Discussion.* The results given in the paper show that after the administration of alloxan sufficient to cause an experimental condition of severe diabetes in rabbits, the coagulability of the blood was very slightly influenced. The clotting time of whole blood and recalcified plasma were slightly prolonged but the other tests that supply more specific information as to the manner in which the mechanism of the coagulation process is influenced, gave normal results. Normal activity of thromboplastin and prothrombin was demonstrated by the normal prothrombin consumption test and the normal prothrombin time. No antithrombin or any other anticoagulant could be demonstrated and the activity of fibrinogen appeared to be normal.

From these results one must deduce that the influence of alloxan diabetes on coagulation is not significant and the prolongation of the coagulation time is probably a non-specific effect, the nature of which remains to be investigated.

*Summary.* Blood of rabbits injected with doses of alloxan monohydrate sufficient to determine an experimental condition of severe diabetes shows a slight delay of the clotting time of both whole blood and recalcified plasma but not accompanied by any appreciable modification of other tests giving more analytical information of the clotting process. The slight hypocoagulability, which is probably non-specific, is not due to the hyperglycemia as it is not corrected by an amount of insulin sufficient to normalize transitorily the blood sugar level.

16704

# Antibiotic Studies on Beta Hemolytic Streptococci. IV. Penicillin Resistance Induced in Mice and Embryonated Eggs.

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Induced *in vivo* penicillin resistance has been demonstrated in only a few species of bacteria. Miller and Bohnhoff[1] were able to induce penicillin resistance in a strain of meningococcus by serial passage in mice when the animals received sub-protective doses of penicillin. After 12 passages, the $PD_{50}$ was increased 100 fold, and the *in vitro* resistance was increased 50 fold. After 60 passages, 170 times as much penicillin was needed to protect 50% of the mice as was required for the first passage. Schmidt and Sesler[2] using two strains of pneumococcus demonstrated a 4 fold rise in penicillin resistance after 7 passages in mice that had been treated with penicillin. In previous reports from this laboratory,[3-5] the development of *in vitro* resistance to penicillin was demonstrated for 28 strains of groups A, B, and C beta hemolytic streptococci. The group A organisms developed resistance very slowly, the group B relatively quickly, and the group C intermediately. The maximum change induced in the group A organisms after 60 transfers was 17 fold, in group B, 190 fold, and in group C, 16 fold. Milzer[6] and his coworkers have recently shown that patients who had received oral penicillin had resistant strains of beta hemolytic streptococci present in their nasopharynx. Typing and grouping were not made, nor were sensitivities determined on the strains before therapy was started. It would be impossible, therefore to say that the organisms that developed resistance were the strains present at the outset.

*Technic.* A total of 6 strains of beta hemolytic streptococci, 2 each of groups A, B, and C were used for the present study. The same strains, designated $S_2$, $S_3$, $T_3$, $T_5$, $U_1$, and $U_2$, were used in our previous reports.[3-5] All were obtained from patients who had received no penicillin therapy.

For mouse passage, 0.03 ml of an 18-hour culture of the organism in veal infusion broth containing 5% defibrinated sheep's blood was injected intracerebrally into each of 6 mice. The animals, in pairs, received 3 injections of penicillin G at 2½-hour intervals. Initially, the total doses arbitrarily assigned were 150, 300, and 450 units given subcutaneously in 0.2 ml amounts. Twenty-four hours after infection, a surviving animal for each of the penicillin dosages was sacrificed, and broth cultures made of the brain and cerebrospinal fluid. The positive culture from the animal receiving the highest quantity of penicillin was used for the next serial passage. The dosage schedule was adjusted for each transfer, so that two animals received the same amount, two a larger amount, and two a smaller amount than that of the mouse from which the culture had been obtained. These changes were approximately 25% for the group A, and up to 100% for the group B and C organisms.

Virulence studies were made on the parent strain, the parent strain after 15 passages in normal mice, and the same strain after 15 passages in animals that had received subprotective doses of penicillin. The technic used for this was the same as that described in a previous communication.[3] The parent and resistant organisms were tested at inter-

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[2] Schmidt, L. H., and Sesler, C. L., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 353.

[3] Gezon, H. M., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 208.

[4] Gezon, H. M., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 212.

[5] Gezon, H. M., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 215.

[6] Milzer, A., Kohn, K. H., and MacLeon, H., *J. Am. Med. Assn.*, 1948, **136**, 536.

TABLE I.
Sterilizing Dosage of Penicillin in Mice Infected Intracerebrally with Streptococci.

| Group | Strain | Daily dosage in units Mean of 5 passages | | |
|---|---|---|---|---|
| | | 1-5 | 6-10 | 11-15 |
| A | $S_2$ | 360 | 540 | 780 |
| A | $S_3$ | 480 | 660 | 630 |
| B | $T_3$ | >1860 | >21,000 | >30,000 |
| B | $T_5$ | >1860 | >21,000 | >30,000 |
| C | $U_1$ | >1860 | >21,000 | >30,000 |
| C | $U_2$ | >1860 | >21,000 | >30,000 |

vals, usually the fifth, tenth, and fifteenth passages, for *in vitro* penicillin sensitivity by the whole plate method.

Fertile hens' eggs, after incubation at 37.5°C for 10-13 days, were infected in the chorio-allantoic space with 0.1 ml of an 18-hour broth culture of streptococcus. This was followed immediately by penicillin G through the same burr hole and at approximately the same depth. Initially, the doses arbitrarily assigned were 0.1, 0.2, and 0.3 units of penicillin per egg in 0.1 ml amounts. Usually 2 eggs were used for each dosage, and 2 as untreated controls. After twenty-four hours incubation all eggs were harvested through the air sac and chorio-allantoic fluid streaked onto blood agar plates. Growth from the egg that had received the largest amount of penicillin was inoculated into broth for the next serial passage. The penicillin dosage was adjusted for each passage, so that 2 eggs received the same amount, 2 approximately 25% more, and 2 approximately 25% less than that of the egg from which the culture had been obtained. *In vitro* sensitivity tests were performed at the fifth, tenth, fifteenth, twentieth, and twenty-fifth passage by the whole plate technic.

*Results.* The results in mice are given in Table I. It may be seen that for the 2 group A organisms no significant increase in the penicillin requirement for bacterial sterilization was found between the first and fifteenth passage in mice.

With group B and group C streptococci, a positive culture was obtained in every instance from the brain of animals receiving the maximum dose of penicillin. A dosage of 30,000 units of penicillin was reached by the ninth passage. It was maintained at this level for the next 7 passages, as this was close to the toxic dose for mice. Positive cultures were also obtained from mice that had been infected with the parent strains and treated with 30,000 units of penicillin. For this reason, the means of the first to fifth and sixth to tenth passages in Table I should not be interpreted as demonstrating an increase in resistance to penicillin. While this may be true, our data do not prove it.

When the group A, B, and C organisms were tested by *in vitro* technics, there was no difference in sensitivity between the parent strains and those obtained after 15 passages in treated mice.

The mouse virulence of all 6 parent strains, the same strains after 15 passages in normal mice, and after 15 passages in penicillin treated mice, were essentially alike.

The results in eggs are given in Table II. For the group A organisms there was no appreciable difference in the amount of penicillin necessary to sterilize the chorio-allantoic fluid in the first and the twenty-fifth passage in both strains studied. In contrast, both group B and C organisms showed a progressively increased resistance to penicillin on serial passages in treated eggs. Approximately 30 to 40 times as much penicillin was required to sterilize the chorio-allantoic fluid for the group B strains on the twenty-fifth passage as was needed for the first. For the group C organisms this difference was 12 and 3 fold respectively. This apparent change in penicillin resistance was not reflected in the *in vitro* sensitivities as all 6 strains maintained their initial sensitivities throughout the 25 serial passages.

*Conclusion.* Two strains of group A streptococci failed to acquire resistance to penicillin after repeated exposures to sublethal amounts either in embryonated hens' eggs or in mice. With 2 strains each of group B and C organisms, however, in eggs there was a definite increase in penicillin resistance. In mice this same increase may have occurred,

TABLE II.
Sterilizing Dosage of Penicillin in Eggs Infected in the Chorio-Allantoic Space with Streptococci.

| Group | Strain | Daily dosage in units Mean of 5 passages 1-5 | 6-10 | 11-15 | 16-20 | 21-25 |
|---|---|---|---|---|---|---|
| A | $S_2$ | 0.19 | 0.13 | 0.17 | 0.24 | 0.28 |
| A | $S_3$ | 0.34 | 0.32 | 0.32 | 0.34 | 0.30 |
| B | $T_3$ | 1.70 | 2.80 | 1.76 | 3.60 | 5.60 |
| B | $T_5$ | 0.42 | 0.66 | 1.52 | 3.80 | 6.60 |
| C | $U_1$ | 0.25 | 0.18 | 0.54 | 0.52 | 0.66 |
| C | $U_2$ | 0.30 | 0.22 | 0.36 | 0.60 | 2.18 |

but we failed to demonstrate it through limitations of the method. The increasing penicillin resistance *in vivo* of the group B and C streptococci was not associated with increasing resistance *in vitro*.

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### 16705

## Drug Sensitivity of Hemolytic Streptococci Isolated from Cases of Scarlet Fever Treated with Penicillin.*

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The development of resistance to penicillin has been observed to occur *in vivo* in strains of *Streptococcus viridans* and *Staphylococcus aureus* during therapy with this agent.[1,2]

Although penicillin has had very wide use in the treatment of group A hemolytic streptococcus infections there have been no reports of the development of strains with lack of sensitivity to this drug which constituted therapeutic problems.

Available information[3–5] would seem to indicate that *Strep. pyogenes* (group A) as encountered in the general population is almost uniformly highly susceptible to penicillin. Hirsch *et al.*,[6] however, have reported the isolation of a strain of this organism which was susceptible only to 0.625 units of penicillin from a patient ill with scarlet fever who had not received any antibiotic therapy. Recently, Milzer *et al.*,[7] reported the occurrence of penicillin-resistant beta hemolytic streptococci in the throat cultures of children with rheumatic fever treated prophylactically with 100,000 units of penicillin orally each day for at least 4 months. Although these strains were resistant to 10 units of penicillin following the period of prophylaxis, none of the streptococci present prior to chemotherapy were available for comparison; nor was the serologic group (group specific carbo-

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* This study was aided by a grant from Schenley Laboratories, New York City.

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hydrate) of the "resistant" strains isolated after treatment determined.

The present report is concerned with a study of the penicillin sensitivity of strains of group A hemolytic streptococci isolated from scarlet fever patients before, during, and following therapy in order to determine whether or not resistance to the antibacterial agent developed during short periods of administration of the drug by either the oral or parenteral route.

*Materials and methods.* The patients from whom the strains hemolytic streptococci described in this paper were obtained had clinical evidence of scarlet fever and were started on penicillin therapy† on admission to the hospital. The total dose of the drug ranged from 1,200,000 to 3,000,000 units intramuscularly or 3,000,000 to 15,000,000 units orally given over a period of 10 days, the interval between each dose being either 3 or 8 hours depending on the schedule employed. Blood levels of penicillin were determined on most patients twice (2nd + 9th days) during treatment and were therapeutically adequate in every instance.

Cultures of the throat and nasopharynx of all patients were made at the time of admission, every day during the 10 days of treatment, and every other day after therapy was discontinued (usually 11 days). If beta-hemolytic streptococci were found in the nasopharynx after the penicillin was stopped, daily culturing was resumed. Characteristic colonies of beta-hemolytic streptococci were isolated from blood-heart infusion-yeast-agar, reinoculated on the same medium and stored at 4°C. All of the strains were grouped by the precipitin technic,[8] and their penicillin sensitivity determined by the method of Rammelkamp[9] using a known penicillin sensitive strain of *strep. pyogene* (R-98) as control.

*Results.* 195 strains of group A beta-hemolytic streptococci isolated from patients before they were treated with penicillin were tested for sensitivity to the drug. Two of the strains were inhibited by 0.0313 unit of penicillin, 75 by 0.0156 unit, 115 by 0.0078 unit, and 3 by 0.0039 unit. This range of sensitivity is essentially in agreement with that previously reported for this organism.[3]

Although penicillin eradicated the beta-hemolytic streptococci from the nasopharynx of most patients with scarlet fever in 48 hours, positive cultures were obtained during treatment on 37 different occasions. The number of hemolytic streptococcal isolations, the day on which the organisms were recovered and their penicillin sensitivity are shown on Table I. These strains had all been in contact with penicillin at the time when their drug sensitivity was determined since they were isolated from cases in which the streptococci failed to disappear despite treatment or from individuals in whom they had disappeared and subsequently recurred probably as the result of exposure to other patients in the ward who still harbored the organisms in the nasopharynx. None of the strains showed a greater tolerance to penicillin than that of the cultures isolated at the time of admission to the hospital before any antibiotic therapy was administered.

Beta-hemolytic streptococci belonging to group A were isolated in 24 instances after penicillin was stopped, the organisms being recovered 1 to 34 days after therapy was halted. Six of these strains were inhibited by 0.0156 unit of penicillin, 16 by 0.0078 unit and 2 by 0.0039 unit. None were penicillin resistant and their presence in the nose or pharynx following a full course of antibiotic administration can probably be accounted for best on the basis of contamination resulting from intimate or remote contact with individuals who were in the early phase of treatment or who had just entered the hospital and had not yet received any penicillin.

*Discussion.* The development of resistant strains of bacteria during contact with various antibiotic agents in use at present is well-

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† The penicillin used in this study was supplied by the Schenley Laboratories, Inc., New York City. The intramuscular penicillin used was the potassium salt of crystalline penicillin G. The oral tablets were crystalline penicillin G potassium salt buffered with calcium carbonate, 50,000 or 100,000 units each.

[8] Swift, H. F., Wilson, A. T., and Lancefield, R. C., *J. Exp. Med.*, 1943, **78**, 127.

[9] Rammelkamp, C. H., Proc. Soc. Exp. Biol. and Med., 1946, **62**, 54.

TABLE I.
Penicillin Sensitivity of Group A Beta-hemolytic Streptococci Isolated During Penicillin Therapy.

| Penicillin sensitivity* | Route of penicillin† administration | Day of treatment and No. of strains of strep. | | | | | | | | | Total isolations |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | |
| .0156 | IM | 3 | 1 | 1 | 1 | 3 | 1 | 2 | 2 | 1 | 15 |
| | PO | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| .0078 | IM | 3 | 1 | 2 | 0 | 0 | 0 | 1 | 1 | 2 | 10 |
| | PO | 2 | 1 | 2 | 0 | 1 | 2 | 1 | 1 | 0 | 10 |
| .0039 | IM | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| | PO | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total | | 10 | 3 | 5 | 1 | 4 | 3 | 4 | 4 | 3 | 37 |

* Quantity which inhibited growth.
† IM—intramuscular; P.O.—oral.

known. The circumstances of the appearance of sulfonamide resistant group A beta-hemolytic streptococci in the Armed Forces has been reviewed elsewhere.[10] The possibility that a similar situation might arise as a result of the extensive use of penicillin has been anticipated but not observed clinically.

Despite the failure of group A beta-hemolytic streptococci to develop proved penicillin resistance *in vivo* during treatment, there is good evidence that this phenomenon can be produced with ease *in vitro*. Weinstein and Tsao[11] made a number of strains of this organism 4 to 32 times more resistant to penicillin by frequent subculture in media containing increasing amounts of the drug; this resistance was only temporary, however, and was made to disappear by frequent subculture in antibiotic-free media. Gezon,[12] using a slightly different technic, reported similar results.

*Summary.* (1) Studies of the penicillin sensitivity of group A hemolytic streptococci isolated from scarlet fever patients before, during, and following penicillin therapy indicate that, with the usual "short" courses of treatment, drug-resistant strains of the organism do not appear.

(2) The likelihood of the development of penicillin resistance in cases of streptococcal infection treated with this agent appears to be very remote.

(3) No penicillin-resistant strains of *Strep. pyogenes* (group A) were recovered in any instance before antibiotic therapy was started.

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[10] Hartman, T. L., and Weinstein, L., *New Eng. J. Med.*, 1948, **238**, 560.

[11] Weinstein, L., and Tsao, C. C. L., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 598.

[12] Gezon, H. M., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 208.

### 16706

## Oxygen Saturation of Sternal Marrow Blood with Special Reference to Pathogenesis of Polycythemia Vera.*

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There are two main theories of the pathogenesis of polycythemia vera. The older theory considers that the disease is neoplastic in nature. The other theory supposes that polycythemia vera, like most forms of experimental and clinical polycythemia, is due in some way to anoxia of the erythropoietic cells of the bone marrow. Because in contrast to secondary polycythemias the percentage oxygen saturation of the arterial blood is normal,[1] the application of this theory to polycythemia vera requires that anoxia be produced by circumstances local to the bone marrow. Reznikoff, Foot and Bethea[2] have described a possible cause of local bone marrow anoxia in certain cases of polycythemia vera in marked narrowing and fibrosis of the arteries and arterioles of that organ. Brilliant support for this concept derives from the recent experiments of Schafer[3] with dogs rendered hypertensive and polycythemic by proprioceptor depressor neurotomy.

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* The expenses of this investigation were defrayed in part by the J. K. Lilly gift to the Harvard Medical School.

† Deceased.

In other cases of polycythemia vera the white blood cell count in the peripheral blood may be elevated with evidence of myeloid immaturity. Likewise, in the bone marrow myeloid hyperactivity and immaturity may be found.[4] Splenomegaly is prominent and the disease process may evolve sooner of later into anemia associated with the features of myelogenous leukemia, non-leukemic myelosis, or even of osteosclerotic anemia.[5,6] Castle[7] has suggested that in this splenomegalic type of polycythemia vera the hyperactivity of the myeloid cells of the bone marrow causes a reduction in the oxygen available to the erythropoietic cells, either because of competitive utilization of the available oxygen by the myeloid cells or because of their mechanical interference with the vascular supply of the erythropoietic cells.

This paper reports an attempt to confirm the validity of this theory by a direct measure of the oxygen saturation and tension of samples of blood removed from the sternal marrow cavity.

*Methods.* Two sets of investigations were carried out. In the first study conducted in 1941 5 cc of blood were withdrawn by gentle suction from the sternal marrow cavity. Thereafter, using conventional methods for handling the sample under oil in an ice bath and employing Wintrobe's dry oxalate mixture as anticoagulant, the carbon dioxide and oxygen contents of the sample as well as its oxygen capacity were determined in duplicate by the Van Slyke manometric combined method[8] using the 0.5 cc technic. In several instances the pH of the marrow blood was determined anaerobically by the glass electrode method using the Beckman instrument.

In the second study begun in 1947 blood samples were obtained by sternal puncture without suction. A sterile 16 gauge needle was washed with sterile heparin solution, the stylet was placed in position and sternal puncture was performed as usual. The patient was then turned on his side so that the needle sloped downward away from its point. The stylet was withdrawn and in many instances blood began slowly to fill the hub of the needle. Whenever no blood appeared, gentle probing with a long stylet usually started a flow. The tip of a 22 gauge needle attached to a tuberculin syringe was then introduced into the hub of the sternal puncture needle and the blood carefully sucked up as it welled into the hub of the needle. The dead space in the needle and the syringe was completely filled with heparin solution, 1 mg per cc.

The blood samples so obtained varied from 0.6 to 1.0 cc in volume. They were handled as described by Roughton and Scholander[9] except that a metal bead replaced the drop of mercury for mixing the blood and heparin solution. Peripheral arterial and venous blood samples were taken immediately after the sternal puncture, using the technic of collection and storage employed with the

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[1] Hitzenberger, K., *Z. f. klin. Med.*, 1934, **126**, 495.

[2] Reznikoff, P., Foot, N. C., and Bethea, J. M., *Am. J. Med. Sci.*, 1935, **189**, 753.

[3] Schafer, P. W., *Ann. Surg.*, 1945, **122**, 1098.

[4] Zadek, I., *Ergebn. d. ges. Med.*, 1927, **10**, 355.

[5] Rosenthal, N., and Bassen, F. A., *Arch. Int. Med.*, 1938, **62**, 903.

[6] Vaughan, J. M., and Harrison, C. V., *J. Path. and Bact.*, 1939, **48**, 339.

[7] Castle, W. B., Chapter on Blood, in *Pathologic Physiology and Mechanisms of Disease,* W. A. Sodeman, editor, in press.

[8] Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, **61**, 523.

[9] Roughton, F. J. W., and Scholander, P. F., *J. Biol. Chem.*, 1943, **148**, 541.

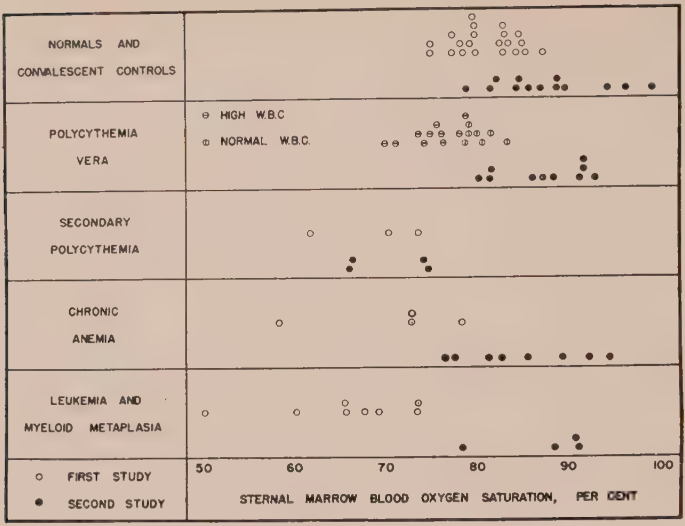

FIG. 1.

Percentage oxygen saturation of samples of blood removed by needle puncture from the sternal marrow cavity of normal subjects, convalescent control patients and patients with polycythemia vera, secondary polycythemia, chronic anemia, and leukemia or myeloid metaplasia. Each symbol represents the result of a single determination. The open dots indicate results from the first study; the solid dots those from the second. The results of the first study on patients with polycythemia vera are divided into two groups, according to whether the white blood cell count of the peripheral blood was normal, 5,000 to 12,900 per cu mm (open dots divided vertically) or high, 17,300 to 40,000 per cu mm (open dots divided horizontally).

marrow blood. Duplicate determinations were made by each of two operators of the oxygen content of the blood samples by the Roughton-Scholander micro method[9] employing 0.04 cc of blood for each analysis. Appropriate allowance was made for the dilution produced by the known volume of heparin solution in the dead space in the needle and syringe. Oxygen capacity was determined by measuring the hemoglobin content of a 0.1 cc sample of blood in an Evelyn photoelectric colorimeter using a factor of 2.58.[10] Control observations were made on the same sample of blood by the Roughton-Scholander, the Van Slyke and photoelectric methods respectively. The results in three such experiments on blood samples with oxygen capacities of 17.3, 17.8 and 18.9 displayed close agreement, the greatest differences being 0.2, 0.3 and 0.4 volumes % respectively. Other control observations on a dummy set-up using partially reduced blood showed no significant exposure of the blood in the hub of the sternal puncture needle to the air before it was sucked into the collecting tuberculin syringe. The accuracy of the Roughton-Scholander technic in our hands when tested on atmospheric air was only slightly beyond the limits claimed for the method by its authors.

*Results.* The results of the observations are plotted in Fig. 1.

*Controls.* In the first studies the oxygen saturation of 21 marrow bloods derived either from normal subjects or from convalescent hospital patients ranged from 75.0 to 87.4 with a mean of 81.1 ± 3.49%. The oxygen capacity of the sternal blood samples from these subjects ranged from 17.3 to 23.6 with an average of 19.8 ± 1.75 volumes %. The pH of the marrow blood in the 8 samples studied ranged from 7.45 to 7.54.

In the second study the oxygen saturation of 13 marrow bloods derived from convalescent hospital patients who were in 2 instances moderately anemic, ranged from 79.0 to 99.2 with a mean of 88.6 ± 5.86%. The oxygen capacity of the arterial blood of these subjects ranged from 15.0 to 21.6 with an average of 18.8 ± 1.99 volumes %.

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[10] Evelyn, K. A., *J. Biol. Chem.*, 1936, **115**, 63.

*Polycythemia Vera.* In the first study, 17 determinations of the oxygen saturation of the sternal marrow blood of 13 patients with polycythemia vera gave readings between 70.1 and 83.4 with a mean of 76.8 ± 3.29%. Of these, 7 determinations were made on 6 patients with normal white blood cell counts in the peripheral blood (5,000 to 12,900 per cu mm. The results showed values between 78.8 and 83.4 with a mean of 80.4 ± 1.64%. Ten determinations made upon 7 patients with elevated white blood cell counts in the peripheral blood (17,300 to 40,000 per cu mm) showed values of 70.1 to 78.9 with a mean of 75.0 ± 2.69%.

In the second study the values obtained for the oxygen saturation of the marrow blood of 10 patients with polycythemia vera ranged from 80.3 to 93.0 with a mean of 87.3 ± 4.49%. In the 3 patients with normal white blood cell counts, the corresponding values were 91.7, 88.5 and 81.7% respectively. The percentage oxygen saturation of the arterial blood samples was within normal limits in all the patients included in this study as well as in 8 other patients with polycythemia vera that exhibited elevated arterial oxygen capacities ranging from 22.8 to 33.0 volumes %.

*Secondary Polycythemia.* Including the observations from both studies 7 determinations of the percentage oxygen saturation of the marrow blood were made in 6 patients with polycythemia secondary either to cardiac or pulmonary disease. In 5 of these patients the oxygen capacity of the arterial blood was elevated, ranging from 22.7 to 30.7 volumes %. The other patient showed a normal oxygen capacity of the arterial blood because of previous venesections. However, in all the arterial blood was definitely unsaturated and carried only from 66.3 to 87.8% of its oxygen capacity. The oxygen saturation of the sternal marrow blood in all instances likewise fell below normal limits with extremes of 61.8 and 74.8%.

*Anemia.* Including observations made in both studies the percentage oxygen saturation of the blood in the sternal marrow of 12 patients with chronic anemia due to various causes was determined. The oxygen capacity of the marrow blood of these patients ranged from 6.0 to 14.2 volumes %. Excluding one patient, with the extremely low value of 58.4%, the oxygen saturation of the marrow bloods ranged from 72.9 to 94.5 and averaged 81.3 ± 6.34. In the 8 patients of the second study in which the determination was made the oxygen saturation of the arterial blood samples was likewise normal.

*Leukemias and Myeloid Metaplasia.* In the first study the oxygen saturation of the marrow blood of 7 cases of leukemia and of myeloid metaplasia was determined, with results ranging from 50.0 to 73.7%. The average value was 65.8 ± 7.8%. However, in the second study the oxygen saturation of the marrow blood of 4 patients ranged between 78.5 and 91.1%, indicating no significant departure from normal values.

*Discussion.* The results obtained and plotted in Fig. 1 do not in general demonstrate a lowered percentage oxygen saturation of sternal marrow blood in polycythemia vera. Also in the few instances in which, in the first study, oxygen tensions were calculated from the oxygen saturation and the pH of the sternal marrow blood according to the methods of Bock and his associates[11] no significant differences between the controls and the patients with polycythemia vera were demonstrated. In the patients with polycythemia secondary to pulmonary or cardiac disease, the percentage oxygen saturation of the marrow blood was distinctly reduced below that of the normals and convalescent controls. However, this was presumably due to the demonstrated unsaturation of the arterial blood.

It is important to note that even in the cases of anemia, where it is generally acknowledged that an anoxic stimulus to blood formation exists, lowered percentage oxygen saturation of the marrow blood was not demonstrable in the present investigation. This result suggests that the failure to find decreased oxygen saturation in the marrow blood of the patients with anemia, and conceivably of those with polycythemia vera, may have been due to failure to obtain samples of blood that truly reflect the environmental conditions of the erythropoietic cells of the bone marrow. Penetration by the needle of the rigid bony cortex surrounding the marrow quite possibly creates a fundamental disturbance of the local pressure relations which, together with the inevitable injury to marrow vessels, may cause a totally abnormal situation with respect to local blood flow.

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[11] Bock, A. V., Dill, D. B., Hurxthal, L. M., Lawrence, J. S., Coolidge, T. C., Dailey, M. E., and Henderson, L. J., *J. Biol. Chem.*, 1927, **73**, 749.

The values for the percentage oxygen saturation of the marrow blood in all patients tested, with a single exception, lay between those of the peripheral arterial and venous blood. Therefore, in the observations reported here as in those of Grant and Root[12] using a somewhat similar technic in dogs, it would appear that samples containing variable proportions of arterial and venous blood were obtained upon marrow puncture. The fact that, in general, in the first study the oxygen saturations of the marrow bloods were lower than in the second suggests that the suction employed in the first study caused a greater proportional backflow of blood from the venous side of the marrow circulation than occurred in the second study in which suction was not used. Because the venous blood leaving the marrow presumably reflects its total metabolic activity, the samples obtained by suction may have more nearly represented its rate of oxygen consumption than did those obtained without suction in the second study.

In the light of this possibility it is interesting to note that there is a suggestion in the first study that the patients with high peripheral white blood cell counts, that is, the cases with myeloid overactivity, tended to show lower percentage oxygen saturations of the marrow blood than did patients with normal or low white blood cell counts. Another feature of interest in the first study is the consistently low values for percentage oxygen saturation obtained in the cases of leukemia and of myeloid metaplasia. Because increased oxygen consumption by the leukocytes of the blood after withdrawal of the sample from the bone marrow could not be demonstrated, these findings suggest that increased oxygen utilization relative to the blood supply was actually occurring in the bone marrow in these patients.

*Summary.* An attempt was made to measure the stimulus to erythropoiesis in the human bone marrow by estimating the percentage oxygen saturation of blood removed from the sternal marrow cavity through a needle inserted through the cortex as in the performance of a sternal needle biopsy. By this means, blood samples were collected without effective contact with air and their oxygen content, capacity and in some instances pH were determined. In general no significant differences were demonstrated between normals, convalescent controls, anemic patients, and patients with polycythemia vera. In patients with secondary polycythemia, the percentage oxygen saturation of the blood removed from the bone marrow was relatively reduced, probably entirely as a result of the manifest unsaturation of the arterial blood. In some patients with leukemia and with myeloid metaplasia and in some patients with polycythemia vera with evidence of excessive myeloid activity, the data suggest an increased local oxygen utilization relative to the blood flow in the bone marrow. However, it was concluded that the technics used were not adequate to demonstrate an anoxic stimulus to increased erythropoiesis even in the marrow of patients with chronic anemia, possibly because of the difficulty of obtaining blood samples satisfactorily representative of the undisturbed environment of the erythropoietic cells.

We desire to express our appreciation to Dr. Benjamin Alexander for permission to carry out the determinations on several of the patients in the second study.

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[12] Grant, W. G., and Root, W. S., *Am. J. Physiol.*, 1937, **150**, 618.

16707

# Incidence of Mammary Tumors in Castrate and Non-Castrate Male Mice Bearing Ovarian Grafts.*

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Although extensive investigations have been carried out determining the state of function of ovaries grafted to castrate, non-castrate and cryptorchid male animals (for review articles see [1,2,3]), there appear to be only 2 reports of the successful production of mammary carcinoma in male mice by this surgical procedure. Murray[4] observed mammary tumors in 7.1% of 210 dba castrate male mice bearing intraperitoneal ovarian grafts while de Jongh and Korteweg[5] report the development of mammary carcinoma in 9 of 16 male mice similarly treated. Since it has been subsequently established that the rodent liver very effectively inactivates estrogenic substances carried through the portal circulation[6,7] and that after castration the adrenals of male mice of at least one strain may produce sufficient estrogen for the development of mammary cancer,[8] it was felt worth while to reinvestigate this problem. The data presented here represent a completion and extension of those previously reported in preliminary form.[9]

*Materials and Methods.* All mice used in these experiments were $F_1$ hybrids between the A and Z ($C_3H$) stocks maintained in this laboratory. As virgin female mice of the reciprocal A × Z cross exhibit a different incidence of mammary carcinoma as well as a different average age of tumor development,[10] approximately equal numbers of animals of the two crosses were employed in each of the groups in these experiments. All surgical procedures were carried out soon after weaning, *i.e.*, when the mice were 4 to 6 weeks of age, after which the animals were housed in wooden boxes, fed Purina Fox Chow *ad libitum,* and inspected once a week for the appearance of subcutaneous tumors. All such tumor masses were studied microscopically.

The method of ovarian transplantation was the same as that previously employed in female mice,[11] consisting of the removal of the ovaries from their encompassing ovarian sac and inserting them deep into the tissue of the axilla through a small skin incision. In our hands this method has proven superior to intraperitoneal implantation in mice since aseptic technics need not be employed as is necessary for intraperitoneal implantation, and the danger of portal vascularization is obviated. From the data obtained with ovarian transplantation into ovariectomized female mice, it would appear that ovaries grafted in this manner function very similar to those remaining in place within the abdomen. Castration was carried out via a transcrotal approach and was done at the

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* Assisted by grants from the Minnesota Division of the American Cancer Society, the Citizens Aid Society of Minneapolis, The Graduate School Cancer Research Fund of the University of Minnesota, and the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

[1] Moore, C. R., and Price, D., *Am. J. Anat.*, 1932, **50**, 13.

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[5] de Jough, S. E., and Korteweg, R., *Acta brev. Neerland.*, 1935, **5**, 126.

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[9] Huseby, R. A., Smith, F. W., and Bittner, J. J., *Cancer Research*, 1946, **6**, 494.

[10] Bittner, J. J., and Huseby, R. A., *Cancer Research*, 1946, **6**, 235.

[11] Huseby, R. A., and Bittner, J. J., Proc. Fourth Inter. Cancer Res. Congress, 1947, in press.

TABLE I.
Summary of Tumor Data from Castrate and Non-castrate Hybrid Males Transplanted with Ovaries. For comparison similar data is given for intact female and ovariectomized ovarian transplanted females of the same hybrid cross.

| | | | Avg age | |
|---|---|---|---|---|
| Group | No. animals/ No. cancerous | % cancerous | Of cancer development, mo. | Non-cancerous deaths, mo. |
| Mammary tumors in $F_1$ hybrid males bearing transplanted $F_1$ hybrid ovaries. | | | | |
| Castrate | 31/27 | 87.1 | 11.7 | 16.9 |
| Non-castrate | 39/1 | 2.5 | 19.4 | 27.0 |

Mammary tumors in normal female mice of the A × Z reciprocal crosses.[10]

| | Virgin | | Breeding | |
|---|---|---|---|---|
| Stock | % cancerous | Avg age of cancer, mo. | % cancerous | Avg age of cancer, mo. |
| $AZF_1$ | 92.5 | 15.2 | 98.0 | 9.7 |
| $ZAF_1$ | 73.2 | 19.1 | 97.6 | 10.1 |
| Average | 82.9 | 16.9 | 97.8 | 9.9 |

Mammary tumors in ovariectomized female mice of the A × Z crosses bearing transplanted hybrid ovaries.[11]

| Stock | % cancerous | Avg age of cancer, mo |
|---|---|---|
| $AZF_1$ | 92.3 | 15.9 |
| $ZAF_1$ | 48.1 | 21.1 |

same time as the ovarian transplantation.

*Results.* Data dealing with mammary tumor development are summarized in Table I. From these it is evident that the presence of the normal testes profoundly affects the development of mammary cancer in male mice bearing ovarian grafts. It is evident, further, that castrate male mice with ovaries develop carcinomas of the mammary gland at a significantly earlier age than do normal virgin females or ovariectomized females bearing ovarian grafts. In fact, in this respect, these castrate male mice bearing ovaries more closely resemble breeding females of this hybrid cross than they do virgin females. This was further emphasized by the fact that there was no difference in the male mice of the two reciprocal crosses as far as either the incidence of tumors or the average age at which these tumors developed.

The ovaries of all the castrate animals that developed mammary tumors and of 12 non-castrate males sacrificed between 362 and 501 days of age were studied employing routine histological procedures. To demonstrate finer cytological details, 2-month-old ovarian grafts carried in castrate and non-castrate males and in ovariectomized female mice were fixed in resorcinol-formol,[12] sectioned at 2 micra, and stained according to the Altmann-Bensley method.

The appearance of the grafts in ovariectomized females was essentially that of normal ovaries. Those recovered from non-castrate males differed mainly in that corpora lutea were absent, and thus the size of these transplants was smaller than that of the transplants carried in ovariectomized females. In the grafts of all mice of this group that were studied, however, numerous normal appearing follicles were present. These ranged in size from primary follicles to ones of moderate size possessing well formed antra. The interstitial cells of these ovaries were somewhat atypical in that with a routine hematoxylin-eosin stain their cytoplasm appeared moderately hypertrophied and more vacuolated than is normal for this strain of mouse. However, the over-all size of the "interstitial organ" was not particularly excessive. In all 20 non-castrate mice sacrificed for histological study, well maintained ovarian

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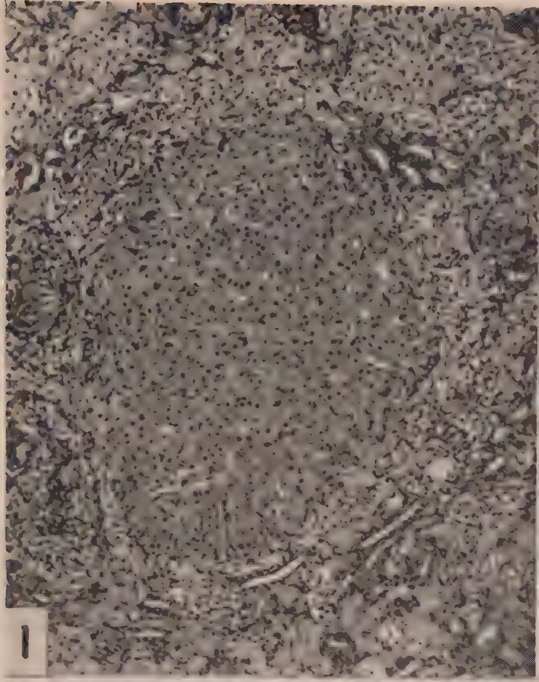
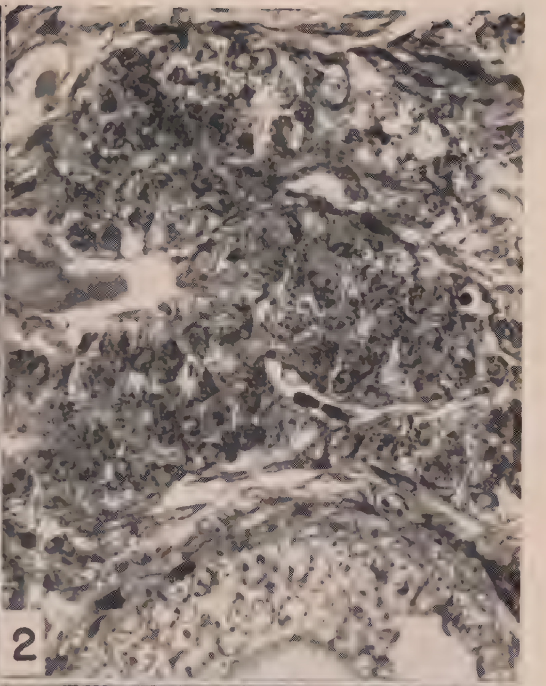
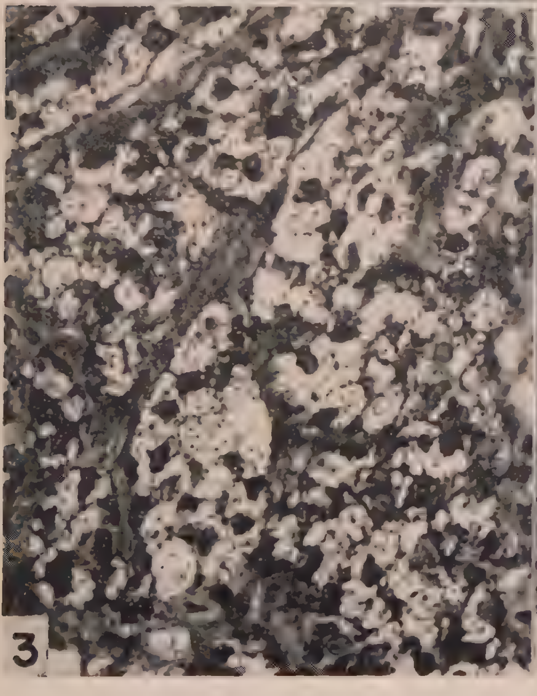
[12] Huseby, R. A., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 122.







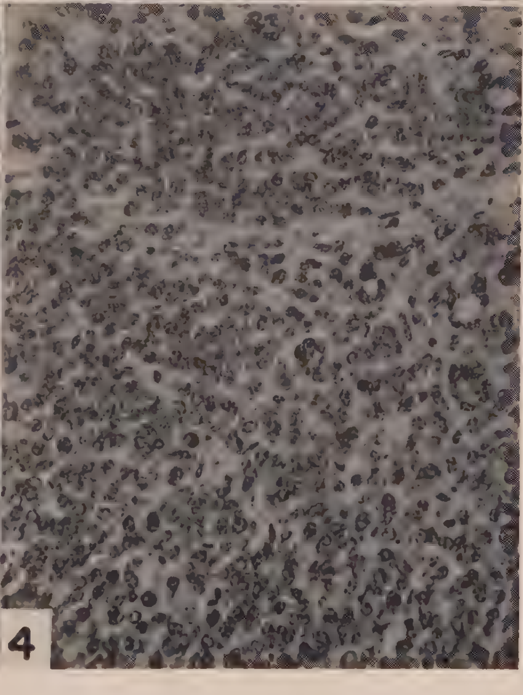


FIG. 1. An area of an ovarian graft carried in a castrate male mouse showing a small corpus luteum. Bouin's fixation and hematoxylin eosin stain. Mag. ×140.

FIG. 2. A group of interstitial cells in an ovarian graft from an ovariectomized female. Note the numerous granules in the cytoplasm and the absence of vacuolization. Resorcinol-formol fixation and Altmann-Bensley stain. Mag. ×800.

FIG. 3. An area of interstitial cells in an ovarian graft carried in a castrate male. Note the extensive vacuolization of the cytoplasm and the paucity of granules. Fixation and staining as in Fig. 2. Mag. ×800.

FIG. 4. An area from a granulosa cell tumor developing in an ovarian graft carried in a non-castrate male. Fixation and staining as in Fig. 1. Mag. ×150.

grafts were found.

The ovarian grafts recovered from castrate male mice differed from those of non-castrate males and of normal females. Although most reports of histological examination of ovarian grafts carried in castrate male animals, mainly in guinea pigs, record a complete absence of corpora lutea, not infrequently in the ovaries of these castrate male mice typical small corpora lutea were encountered (Fig. 1). The ovaries of $F_1$ hybrid females of the A × Z cross more closely resemble those of the Z parent in that corpora lutea are retained for a considerable time and thus normally this element makes up a large portion of the ovarian mass during the reproductive period in the animal's life.[11] It is evident, therefore, that although corpora lutea appeared not infrequently in the ovarian grafts of these castrate males, they were by no means as prevalent as in ovaries maintained in female animals. Conversely, however, the interstitial tissue of these transplanted ovaries was greatly hypertrophied and the cytoplasm of the individual interstitial cell possessed many large vacuoles. Follicles were numerous, appeared cytologically normal, but in general more large follicles with medium to large sized antra were evident than is the case in normal ovaries in female mice. In size these ovarian grafts considerably exceeded those of the non-castrate males and equaled or slightly exceeded those in ovariectomized females. Weights were not taken because of the difficulty of dissecting the ovaries completely free of surrounding connective tissue.

The detailed cytological study of the ovarian grafts revealed the follicular elements to be normal in all instances as were the cells of the large corpora lutea in the female grafts and of the small corpora lutea in the grafts of the castrate males. The interstitial cells of the 3 groups of ovaries differed greatly, however. In the female grafts studied, as well as in normal females of this cross, the cytoplasm of the interstitial cells was generally crowded with rather large, brilliantly fucsinophilic granules (Fig. 2). In the non-castrate males some cells possessed a large number of these granules while the cytoplasm of others was rather extensively vacuolated with a loss of granulation. The cytoplasm of the interstitial cells of the ovaries in the castrate males was uniformly hypertrophied and extensively vacuolated with but few fucsinophilic granules remaining (Fig. 3).

These histological studies would suggest that the ovaries in the castrate males were subjected to a greater pituitary stimulation than were those in the non-castrate group.[13] In the majority of cases the adrenals of castrate tumor bearing animals were studied also, and no example of adrenal hyperplasia was noted.

In view of the great difference in the development of mammary carcinoma in the two groups of animals of this experiment, a histological study of their mammary glands was made employing the whole mount technic. The glands of the castrate males were found to be extensively developed with large ducts extending throughout the major portion of the various fat pads. In addition to ductular growth there were many areas of alveolar development, some of which formed typical pre-cancerous lesions while others appeared as more normal lobules in the early stages of secretion, *i.e.*, similar to those seen in normal female glands about the time of parturition. In some animals the mammary ducts were filled with a white, milk-like secretion. As has been observed by others, all 10 glands rarely develop in male mice receiving estrogenic stimulation. As contrasted to this picture, no definite example of mammary gland development was found in the non-castrate males. In some a few fine thread-like ducts could be seen extending a considerable distance into a fat pad, particularly in the number two position, but such development is not infrequent in normal male mice of this hybrid cross. Neither lateral buds nor alveoli were seen in these glands. Examination of the glands of the one non-castrate male that developed a mammary adenocarcinoma showed no more mammary growth than was seen in the other animals of the group.

It seemed possible to test the function of the well preserved ovarian grafts of non-

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[13] Pfeiffer, C. A., and Hooker, C. W., *Anat. Rec.*, 1942, **83**, 543.

TABLE II.
Development of Uterine and Vaginal Grafts and Mammary Glands in Mice 2 Months After Ovarian Transplantation.

| Ovaries transplanted to | Uterine grafts | Vaginal grafts | Mammary glands |
|---|---|---|---|
| Ovariectomized females | Developed.<br>Avg 6.0 × 4.3 mm | Cyclic | Developing |
| Non-castrate males | Developed.<br>Avg 5.3 × 3.9 mm | No evidence of estrogenic stimulation* | Little or no development |
| Castrate males | Developed.<br>Avg 8.0 × 7.0 mm | Non-cyclic cornified | Developing |

* There may be a beginning mucinification of the epithelium suggesting estrogen-androgen cooperation.

castrate males by grafting bits of uteri and vaginae into the subcutaneous tissue of such mice. As a preliminary experiment, the uteri and vaginae of young $F_1$ hybrid females were removed, each uterine horn was split longitudinally, and each vagina was cut transversely into two narrow segments. Single uterine and vaginal fragments were then transplanted into the subcutaneous tissue (in either the axilla or groin) of several intact male and female $F_1$ hybrid mice. Two months later the recipient animals were sacrificed, and the grafts studied histologically.

The uterine grafts carried in female mice formed large thin-walled cysts lined with typical uterine epithelium. Because of the pressure exerted on the cyst wall by the accumulating fluid, the epithelial cells were generally rather low columnar or cuboidal in form. Only in areas of papillary infoldings were the characteristic tall columnar cells with significant amounts of underlying lamina propria found. In the intact male animals the uterine grafts had been reconstituted so as to form small cylindrical bodies very similar to the intact donor uteri before being split longitudinally. There was no tendency for fluid accumulation so that the normal uterine relationships were disturbed very little. Morphologically these grafts closely resembled uteri of castrate female mice except for a slightly "looser" appearance of the lamina propria.

Vaginal grafts were retained as cysts also. The epithelial lining seen in the grafts in the female animals faithfully reproduced that of the *in situ* vaginal epithelium, and the cyst lumen was filled with alternate layers of leukocytes and desquamated cornified epithelial cells. On the other hand, the epithelium of the vaginal grafts maintained in intact male mice consisted entirely of two layers of epithelium similar to that found in castrate female mice and the luminal contents were devoid of cellular components. This method of uterine and vaginal transplantation showed promise then of shedding light on the state of function of ovarian grafts carried in male animals.

Groups of 8 weanling hybrid mice were set up as follows: non-castrate males, castrate males, and ovariectomized females. Into each animal was transplanted 2 ovaries, a vaginal, and a uterine fragment. Two months later the animals were sacrificed, the uterine and vaginal grafts measured (2 diameters at right angles to one another), and histological preparations of all grafts studied. A summary of these findings is given in Table II. It is evident from the size and histology of the uterine grafts in the non-castrate male group that the ovaries of these animals were producing estrogenic substances in appreciable amounts, although in all probability, in lesser quantities than produced by the ovaries of the castrate group. In view of this, the morphology of the vaginal grafts is of considerable interest. The epithelium of the grafts in the non-castrate animals was formed throughout by only 2 layers of cells much the same as in the vaginal grafts of intact male mice without ovarian transplants. The only indication of development was a tendency for the luminal layer of cells to be low columnar in form and to possess an increased amount of clear cytoplasm. This appearance

TABLE III.
Development of Vaginal Grafts and Mammary Glands in Castrate and Non-castrate Male Mice After Injection of Various Doses of Estrone for 3 Weeks.

| Amt of estrone | | Vaginal mucosa | Mammary gland |
|---|---|---|---|
| .08 $\gamma$ in 2 days twice per week | Castrate | Partial cornification with considerable mucinification | No development |
| | Non-castrate | No evidence of estrogen activity | No development |
| .05 $\gamma$ per day | Castrate | Cornification with some areas of mucinification | Only 1 of 4 animals showed mammary development |
| | Non-castrate | Epithelial development mainly mucinification | Little or no development |
| .15 $\gamma$ per day | Castrate | Completely cornified | Good mammary development |
| | Non-castrate | Cornification with some areas of mucinification | Little or no development |

is suggestive of beginning mucinification which Korenchevsky and Hall[14] have shown to result from the cooperative action of estrogens and androgens or progesterone. The vaginal grafts in the castrate male animals are also worthy of some description, for although they appeared extensively stimulated, there was no indication of estrous cycling. The epithelial histology was the same in all 8 grafts; however, there was considerable variation from area to area within each graft. While most areas showed complete or almost complete cornification, others exhibited only partial cornification with leukocytes passing between the epithelial elements. The lumina were filled uniformly with desquamated cornified epithelium mixed with some leukocytes, but with no indication of stratification as was seen in the grafts carried in normal female mice. This "mixed" vaginal histology is similar to that seen in female mice subjected to a constant stimulation with estrogen.

Although the difference in uterine and vaginal-mammary development in the non-castrate male mice might reflect only a difference in tissue sensitivity to estrogen, it might also reflect an antagonism between the two classes of sex hormones with regard to the vaginal and mammary epithelium. This latter possibility was investigated by transplanting a group of hybrid male mice with uterine and vaginal fragments and allowing 2 weeks to elapse for vascularization of the grafts. Half of the mice were then castrated, and groups of castrate and non-castrate animals were injected with graded doses of estrone with 4 castrate and 4 non-castrate animals receiving each dose level. Injections were begun the day following castration in order to minimize the possibility of an increased estrogen production by the adrenals of the castrate animals. The estrone was injected subcutaneously in aqueous solution, the daily dose being equally divided between a morning and an afternoon injection. Animals at the lowest dosage level received 0.4 $\gamma$ a day on 2 successive days and for 4 days of each week. The other groups received 0.05 and 0.15 $\gamma$ daily 7 days a week. Injections were continued for 3 weeks, and the animals were autopsied within 6 hours of their last injection. A summary of the results is given in Table III. The data on the uterine grafts are not included as measurements on such a small number in each group could not be considered conclusive. It can be said, however, that at all dosage levels the uterine grafts became cystic and that there was no consistent difference in size between the grafts of castrate and non-castrate animals receiving the same amount of estrone, although the grafts of the animals receiving 0.15 $\gamma$ of estrone generally exceeded those of the other 2 groups.

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[14] Korenchevsky, V., and Hall K., *J. Path. and Bact.*, 1937, **45**, 681.

The study of the vaginal grafts and mammary glands suggest two major points. First, there is a considerable difference in the sensitivity threshold of these 2 tissues to estrone. Second, there would appear to be some antagonism between testicular hormone and estrone. To establish this latter conclusion with certainty, however, differences in adrenal function must be completely ruled out, although under the conditions of this experiment significant differences in this respect seem unlikely. Although the state of function of the pituitary does not appear to alter the vaginal response to estrogen,[15] it has been found that mammary gland growth is dependent upon a functional pituitary gland (for review see [16]). In order to be certain that the amounts of estrone injected had not caused a sufficiently severe hypophyseal suppression to inhibit mammary growth in the non-castrate males, the testes, ventral prostate, and seminal vesicles of each animal were studied. In all instances these organs were grossly and microscopically indistinguishable from those of normal intact males. Since, in our experience, doses of estrogens considerably lower than that required to cause "stunting" of the mammary gland produce easily demonstrable reduction in the production of pituitary gonadotropin, it would appear that in these estrone injected non-castrate mice pituitary suppression was not the factor responsible for the reduced response of the mammary glands.

The non-castrate males bearing ovarian grafts lived significantly longer than do virgin female mice of this hybrid cross (those lacking the milk agent so that no mammary cancer developed.[10]) It is, therefore, of interest to record that in 4 of these animals typical granulosa cell tumors developed in the area of the transplanted ovary (Fig. 4). These tumors developed when the mice were very old: 24.9, 25.4, 30.2, and 32.2 months of age. In none of these 4 animals was there evidence of high estrogen levels since the mammae were no more developed than in other animals of the group.

Since it has been suggested that an increased pituitary gonadotropin level is responsible for ovarian tumor formation in the Biskind-Biskind type of experiment,[17,18] a group of 35 $F_1$ hybrid males lacking the milk agent were castrated and transplanted with ovaries in the axillary position. The average life of these males was 24.6 months, but only two granulosa cell tumors were observed: at 19.3 and 23.3 months. Although these tumors developed somewhat earlier in life than did those in the non-castrate males, it is evident that no increase in the frequency of this tumor was brought about by castration. One of these tumors was transplanted to the subcutaneous tissue of other $F_1$ hybrid mice and was observed to grow rapidly in intact as well as in castrate males and in intact females. No evidence of estrogen production by these transplants could be found.

As there are no data available on the spontaneous occurrence of granulosa cell tumors of the ovary in normal female mice of this hybrid cross to use for comparative purposes, the ovaries transplanted to oöphorectomized females of this strain[11] were reviewed. For this comparison only mice living to be 700 days of age or older were included. There were 18 female animals bearing hybrid grafts and living to an average age of 26.0 months available for study. In these only one atypical ovary possessing an overgrowth of granulosa cells was encountered (age 28.7 months). This is to be compared with the non-castrate male group bearing ovarian grafts in which 21 lived beyond 700 days of age (average age of death 28.0 months) and in which 4 typical granulosa cell tumors developed. Because of the small number of tumors observed and the somewhat longer average life of the male group, no statement as to the relative frequency of this type of tumor in the two groups seems justified.

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[15] Smith, P. E., *Am. J. Anat.*, 1930, **45**, 205.

[16] Huseby, R. A., Ball, Z. B., and Visscher, M. B., *Cancer Research*, 1945, **5**, 40.

[17] Biskind, M. S., and Biskind, G. R., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 176.

[18] Li, M. H., and Gardner, W. U., *Science*, 1947, **105**, 13.

It seems of value, also, to record the occurrence of ovarian tumors developing in the ovaries of the two parent strains when transplanted to ovariectomized $F_1$ hybrid females. There were 11 hybrid females bearing Z or $C_3H$ ovaries living beyond 700 days of age, and in these one mixed ductular-granulosa cell tumor was found (age 24.2 months). In 13 hybrid females past 675 days of age and bearing strain A ovaries, one typical luteoma (23.5 months) and two granulosa cell tumors (22.5 and 25.4 months) were encountered.

*Discussion.* It has been previously recorded that castrate male guinea pigs bearing ovarian grafts exhibit a greater mammary gland development than do normal virgin females (for review see [2]). The present experiments with mice show that castrate males with ovarian grafts developed mammary cancers earlier than do normal virgin females of this hybrid cross, in fact the average age of tumor development approached that seen in similar breeding females. Although Smelser[2] found some indication of cyclic pituitary function in castrate, ovarian grafted male guinea pigs, our experience with vaginal grafts in castrate, ovarian grafted male mice suggests that the gonadotropin stimulation from these male pituitaries is at all times such that sufficient ovarian estrogen is produced to extensively cornify the vaginal epithelium. Although the largest part of the epithelium of these grafts was completely cornified, areas were encountered in all in which leukocytes could be seen passing through an incompletely cornified epithelium. This "mixed" picture is consistent with that seen in vaginae subjected to long periods of continuous estrogen stimulation.

The failure of the mammal to develop in the non-castrate males bearing ovarian grafts is in agreement with the observations of Smelser in guinea pigs. It is, however, at variance with those of Gardner[3] who employed intratesticular grafts in CBA mice. This may, of course, be a strain difference. However, Gardner's photomicrographs show considerable tubular damage in the testes due to the ovarian transplant. It seems possible that this might explain the difference since Smelser observed mammary development in cryptorchid guinea pigs bearing intranephric ovarian grafts in which the androgen production by the cryptorchid testes was adequate to maintain the secondary sex glands but in which tubular alterations were extensive because of the intra-abdominal position of the testes. Further experimentation is necessary to establish or reject such an explanation.

The results of the experiments reported here designed to test the function of the ovarian grafts in the non-castrate male animals would seem to indicate that significant amounts of estrogen were being produced, but that the quantities were smaller than those produced by the ovarian grafts carried in castrate males. Any estimation of relative amounts of estrogen production by the grafts in these two groups of animals seems unwarranted since the picture appears to be further complicated by antagonism between estrogen and androgen with regard to vaginal and mammary gland development in the non-castrate group.

An estrogen-androgen antagonism for both the vaginal and mammary epithelium seems fairly well established by the results of the last experiment reported here. At the lowest dose level of estrone, the vaginal grafts in the castrate males showed definite histological evidence of stimulation while in the non-castrate group no demonstrable epithelial development was seen. At the higher dose levels, the cooperative action of the two hormones as described by Korenchevsky and Hall[14] is also clearly demonstrated. Robson[19] has suggested such an antagonism, but since he used only smears to follow the vaginal response one does not know whether he observed true antagonism or the co-operative effects resulting in epithelial mucinification which would also produce a "negative" vaginal smear. Gardner[20] has also shown this antagonism for the mammary gland by injecting estradiol benzoate and testosterone propionate concurrently. The possibility that the antagonism in the mammae is due to pituitary suppression resulting in mammary gland "stunting" seems to be ruled out in our experiment in which relatively small doses of estrogen and the androgen from functioning testes were employed.

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[19] Robson, J. M., *J. Physiol.*, 1937, **90**, 15.

[20] Gardner, W. U., *Cancer Research*, 1946, **6**, 493.

*Conclusion.* Castrate male mice of the $F_1$ hybrid generation between the A and Z stocks bearing subcutaneous ovarian grafts developed mammary cancer more readily than do virgin female mice of this cross. Only 1 of 39 non-castrate males bearing ovarian grafts develop mammary carcinoma as little mammary development occurred. Additional experiments seemed to indicate that the ovaries in the non-castrate male mice were producing an appreciable amount of estrogen although measurably less than those carried in castrate males. There appeared, also, to be an antagonism between estrogens and androgens as far as stimulation of the vaginal and mammary epithelium is concerned. Granulosa cell tumors occasionally occurred in ovarian grafts of old male and female mice of this hybrid cross.

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## 16708 P

# The Effect of 11-Desoxycorticosterone Acetate Upon the Glycosuria of Partially Depancreatized Rats.

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This study shows that large doses of 11-desoxycorticosterone acetate cause an exacerbation of the diabetes of partially depancreatized, force-fed rats.

*Methods.* Male rats of the Sprague-Dawley strain were maintained on Archer Dog Pellets until they reached a weight of 300 g. Ten rats were depancreatized by the method of Ingle and Griffith.[1] After diabetes was established all of the animals were placed in metabolism cages and were fed a medium carbohydrate diet made according to Table I. All of the animals were force-fed by stomach tube each morning (8:30 to 9:15 A.M.) and afternoon (4:15 to 5:00 P. M.). The technics and diets were modifications of those described by Reinecke, Ball and Samuels.[2] During the period of adaptation to force feeding, the amount of diet was increased gradually to prevent "food shock." A full feeding of 26 cc of diet per rat per day was reached on the 5th day.

TABLE I.
Medium Carbohydrate Diet.

| Constituent | G |
|---|---|
| Cellu flour (Chicago Dietetic Supply) | 120 |
| Osborne & Mendel salt mixture | 40 |
| Dried yeast (Pabst) | 100 |
| Wheat germ oil | 10 |
| Cod liver oil | 10 |
| Vitamin K (2-methyl-1,4-naphthoquinone) | 100 mg |
| Mazola oil | 200 |
| Casein (Labco) | 160 |
| Starch | 200 |
| Dextrin | 190 |
| Sucrose | 200 |
| Water to make total of | 2000 cc |

The animals were kept at a temperature of 74° to 78°F and a humidity of 30 to 35% of saturation. Twenty-four-hour samples of urine were collected at the same hour (8:00 to 8:30 A.M.) and were preserved with thymol. Urine glucose was determined by the method of Benedict.[3]

The 11-desoxycorticosterone acetate (Cortate, Schering) was made up in sesame oil at 5 mg per cc. It was given by subcutaneous in-

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[1] Ingle, D. J., and Griffith, J. Q., Chapter 16, The Rat in Laboratory Investigation, J. B. Lippincott Co., Philadelphia, 1942.

[2] Reinecke, R. M., Ball, H. A., and Samuels, L. T., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 44.

[3] Benedict, S. R., *J.A.M.A.*, 1911, **57**, 1193.

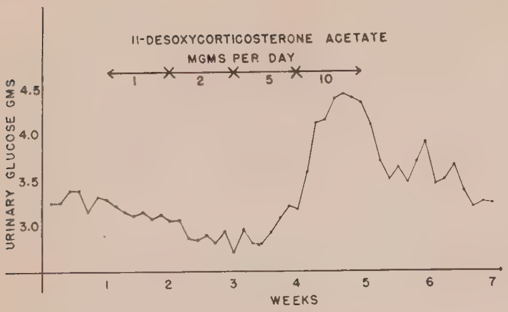

Fig. 1.

Effect of 11-desoxycorticosterone acetate upon the average level of urinary glucose for 10 partially depancreatized rats.

jection in divided doses each morning and late afternoon.

*Experiments and results.* Ten mildly diabetic rats were observed for periods of several weeks prior to the beginning of the experiment. All of the rats received doses of 1, 2, 5 and 10 mg of 11-desoxycorticosterone acetate for periods of 7 days per dose. At the completion of the injection periods the rats were observed during an additional control period of 14 days before the experiment was terminated. Several days elapsed between the beginning of the injection period for each rat and those tested subsequently so that possible unknown variables related to time were less likely to cause fortuitous shifts in the average level of glycosuria. This is an important control for on many occasions we have observed small but significant spontaneous shifts in the level of glycosuria in most or all of a series of diabetic animals under rigid experimental conditions and constant food intake. The results are shown in Fig. 1. During the administration of 1 and 2 mg of the steroid per day there was an average decrease in the level of urinary glucose representing a change in 7 of the 10 experimental animals. When 10 mg per day of 11-desoxycorticosterone acetate were given, there was an exacerbation of the glycosuria in each of the 10 rats which decreased to pre-injection levels following the withdrawal of the hormone.

*Discussion.* The decrease in glycosuria which occurred in most of the rats during the administration of 1 and 2 mg per day of the steroid may have been due to the compensatory suppression of the secretion of the 11-oxy steroids by the adrenal cortices according to the hypothesis of Selye and Dosne.[4] However, the changes were not sufficiently striking to permit the definite conclusion that the decrease was caused by the administration of the steroid. The exacerbation of glycosuria during the injection of 10 mg per day of the steroid was clearly significant and supports the conclusion that 11-desoxycorticosterone does affect carbohydrate metabolism when given in large doses.

*Summary.* Ten mildly diabetic force-fed rats showed an average decrease in the level of urinary glucose during the administration of 1 and 2 mg of 11-desoxycorticosterone acetate per day. When the dosage was increased to 10 mg of the steroid per day the glycosuria was exacerbated in all of the animals and decreased to the pre-injection level when the administration of the steroid was stopped.

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[4] Selye, H., and Dosne, C., *Endocrinology*, 1942, **30**, 581.

### 16709

# Inhibition of Brain Respiration by Ethyl Alcohol at Varied Temperature Levels.

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Jowett[1] has reported that given concentrations of ethyl alcohol produce less inhibition of rat brain respiration at 34°C than at 39°C. The experiments reported here were carried out in order to study the inhibitory effect of ethyl alcohol over a wider range of temperature and concentration than had hitherto been investigated.

*Methods.* The oxygen consumption of cerebral cortex slices from adult albino rats was measured *in vitro* by the direct method of Warburg at 37.7°C, 30°C and 20°C. The tissue slices were weighed on a micro torsion balance and transferred to respirometer vessels containing Ringer's solution[2] buffered to pH 7.25 with 0.01 M phosphate and containing 0.011 M glucose. The gas phase was oxygen. Ethyl alcohol in appropriate concentration, in one-tenth the total fluid volume, was added from the side arm of the vessels at the end of a 10 minute period of thermoequilibration. Rates of oxygen consumption are expressed as ml of oxygen consumed per mg initial dry weight (determined on aliquots) per hour ($QO_2$). Complete details of the experimental procedure have been given elsewhere.[3]

*Results and discussion.* The rates of oxygen consumption of rat brain slices in the presence of various concentrations of ethyl alcohol at 37.7°C, 30°C and 20°C are given in Table I. The values of $QO_2$ at 37.7°C are the means of 4 determinations while those at other temperatures are single determinations. Additional values of $QO_2$ (not given in the table) at 37.7°C for the period 120-180 minutes after addition of alcohol are 11.10 at 0.95% alcohol, 10.43 at 2.85%, 6.91 at 4.75% and 2.16 at 6.65%.

The inhibition of brain respiration produced by ethyl alcohol has been reported by Jowett[1] to become progressively greater with time. It may be seen from the table that the progressive inhibition is more marked at 37.7°C than at 30°C. At 20°C this decrease in rate of oxygen consumption with time does not occur within 3 hours. At all of these temperatures the $QO_2$ of control brain slices, without addition of alcohol, remains constant for at least 5 hours.

In order to minimize the effect of the progressive inhibition in the presence of alcohol, the $QO_2$ for the period 120-180 minutes after addition of the inhibitor has been selected for purposes of comparison. It is apparent that the minimum effective inhibitory concentration of alcohol increases with decrease in temperature. It is approximately 2.5% at 37.7°C, 6% at 30°C and 7% at 20°C. The percentage inhibition produced by a given concentration of alcohol is greater at 37.7°C than at the lower temperatures. With 9.5% alcohol, for example, the inhibition is 91% at 37.7°C., 64% at 30°C and 21% at 20°C. The absolute level of oxygen consumption in the presence of 9.5% alcohol is remarkably similar at the 3 different temperatures.

At 37.7°C the inhibitory effect of higher concentrations of alcohol was studied. With 23.73% alcohol the $QO_2$ was 0.93 and further decrease did not occur over a period of 3 hours. The oxygen consumption was completely abolished by 47.5% alcohol. There is thus a large range of alcohol concentration, from about 8% to well over 25%, within which the rate of oxygen consumption is approximately constant. This fraction of oxygen consumption of brain, amounting to about 10% of the control rate at 37.7°C, has been

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[1] Jowett, M., *J. Physiol.*, 1938, **92**, 322.

[2] Dickens, F., and Greville, G. D., *Biochem. J.*, 1935, **29**, 1468.

[3] Fuhrman, F. A., and Field, J., 2nd., *J. Pharm. Exp. Therap.*, 1943, **77**, 229.

TABLE I.
Effect of Time and Temperature on Inhibition of Rat Brain Oxygen Consumption by Ethyl Alcohol. See text from experimental details. Oxygen consumption expressed as $Q_{O_2}$ for time periods given.

| Temp. °C | Time after addition of alcohol, min. | Conc. of ethyl alcohol—% by volume | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 1.90 | 3.80 | 5.70 | 7.60 | 9.50 |
| 37.7 | 30- 60 | 11.02 | 11.74 | 10.82 | 9.89 | 8.24 | 3.70 |
| | 60-120 | 11.02 | 11.74 | 9.68 | 7.82 | 3.61 | 1.39 |
| | 120-180 | 11.02 | 11.74 | 8.14 | 4.58 | 1.03 | 1.03 |
| 30.0 | 30- 60 | 5.25 | 6.59 | 5.30 | 5.46 | 4.74 | 4.02 |
| | 60-120 | 5.25 | 6.08 | 5.30 | 5.46 | 4.17 | 2.68 |
| | 120-180 | 5.25 | 5.87 | 5.30 | 5.46 | 3.45 | 1.91 |
| 20.0 | 30-180 (Constant) | 1.96 | 2.27 | 1.96 | 1.85 | 1.55 | 1.55 |

termed the "inhibitor stable" respiration. It has been found to be of approximately equal magnitude in brain slices maintained for over 3 hours without added substrate[4] and in the presence of a number of other inhibitors.[3,5-7]

It may be seen from Table I that low concentrations of alcohol appeared to produce an increase in the rate of oxygen consumption at all 3 temperatures. The effective concentration for this augmentation was about 2%. A similar increase in brain $QO_2$ with alcohol concentrations of from 1.6 to 3.2% has been reported by Levy and Olszycka.[8] It is possible that this increased rate of oxygen consumption is due to alcohol metabolism by brain tissue.[9]

*Summary.* The inhibition of oxygen consumption of rat cerebral cortex slices by ethyl alcohol was studied at 37.7°C, 30°C and 20° C. The inhibition becomes progressively greater with time at 37.7°C; such progressive inhibition is less marked at 30°C and is absent at 20°C. The minimum effective inhibitory concentration increases with decrease in temperature. The percentage inhibition with a given concentration of alcohol decreases with decrease in temperature.

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[4] Fuhrman, F. A., and Field, J., 2nd., unpublished results.

[5] Fuhrman, F. A., and Field, J., 2nd., *J. Cell. and Comp. Physiol.*, 1942, **19**, 351.

[6] Fuhrman, F. A., and Field, J., 2nd., *J. Pharm. Exp. Therap.*, 1943, **77**, 392.

[7] Fuhrman, F. A., *Abst. of Dissertations, Stanford University*, 1943, **19**, 3.

[8] Levy, J., and Olszycka, L., *Compt. rend. Soc. Biol.*, 1940, **133**, 370.

[9] Himwich, H. E., Nahum, L. H., Rakieten, N., Fazekas, J. F., DuBois, D., and Gildea, E. F., *J.A.M.A.*, 1933, **100**, 651.

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### 16710

## Hemagglutination by Botulinal Toxin.

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In the course of studies of the properties of type A botulinal toxin we have observed that the addition of toxin to red cell suspensions results in agglutination. Crystalline and amorphous preparations of toxin have proven to be equally capable of causing hemagglutination. Observations for agglutination have been positive with red cells from all animal species tested (chicken, guinea pig, rabbit, sheep, man.)

TABLE I.
Influence of Temperature on Hemagglutination Titer.

| Dilution | 0* | 2 | 4 | 8 | 16 | 32 | 64 | 128 | Control |
|---|---|---|---|---|---|---|---|---|---|
| Sheep cells: | | | | | | | | | |
| 0°C | + | + | + | sl+ | 0 | 0 | 0 | 0 | 0 |
| 45°C | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Chicken cells: | | | | | | | | | |
| 0°C | + | + | + | + | sl+ | 0 | 0 | 0 | 0 |
| 45°C | + | + | sl+ | sl+ | 0 | 0 | sl+ | 0 | 0 |

Each tube contained a mixture of 1 ml of diluted toxin and 1 ml of red cells. Saline was used as diluent. There were $131 \times 10^6$ cells per tube in the case of the sheep cells and $38 \times 10^6$ cells per tube in the case of the chicken cells. These numbers of cells were chosen so as to give about 3.45 times as many sheep cells as chicken cells which is about the reported difference in surface area of these cells. Agglutination was read by "pattern" test.

* This tube contained about 25,000 mouse MLD, so that as little as 1-3,000 MLD are giving macroscopic evidences of agglutination in the endpoint tubes.

The agglutination can be followed either microscopically or macroscopically. Methods in current use for observation of virus hemagglutination$^{1-3}$ are applicable to studies with the toxin. A most interesting difference from the experience with viruses is that the toxin does not appear to be taken up by the clumped red cells under the conditions studied: chicken and sheep cells at 0°C and room temperature; guinea pig cells at 30°C. The suspending menstruum suffers no reduction in toxic titre by mouse titration when agglutinated cells are centrifuged off. The centrifuged red cells themselves show a low (less than 99% of the toxin) and irregular order of toxicity which in separate tests has been reduced or abolished by washing with saline. The toxicity can be as logically attributed to the cells being contaminated with slight quantities of toxin by reason of the residual fluid wetting the cells after centrifugation as to a real adsorption phenomenon.

The addition of formaldehyde to toxin solutions results in diminished toxicity and hemagglutination. Other conditions that reduce the toxic titre such as aging in a dilute solution of salt, also decrease the hemagglutination titre. A corollary observation is that the employment of a peptone-saline mixture as diluent in place of the use of saline alone often gives an increased hemagglutination titre. Such a protective colloid effect is not unexpected in view of the readiness with which purified toxin is biologically inactivated by surface denaturation. These observations make it highly unlikely that a non-toxic fraction can split off from the toxin molecule and cause hemagglutination independent of a toxic fraction which remains behind in solution.

The hemagglutination reaction is specifically prevented by type A antitoxin. Commercially prepared monovalent types A, B, and C horse antitoxins have been tested. The order of adding the reagents namely, toxin, antitoxin and red cells does not affect the inhibition by antitoxin. These observations provide conclusive proof that the hemagglutinating activity of the toxin preparations used was due to the toxin itself rather than to contaminating substances. Because ribonucleic acid is a major foreign material that accompanies the toxin fractions during several stages of purification, separate tests for hemagglutination by purified ribonucleic acid were performed. The ribonucleic acid was isolated from cultures of the same medium and strain of organisms as used for toxin production. Purified yeast nucleic acid was also tested. These nucleic acids did not agglutinate red cells.

The titre of a given mixture of toxin and red cells was found to vary with the temperature. But the influence of temperature was itself conditioned by the animal source of the red cells. Table I illustrates these findings. In attempting to compare red cells from different species, sheep and chicken cells were studied since among the common laboratory

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[1] Hirst, G. K., *J. Exp. Med.*, 1942, **76**, 195.

[2] Hirst, G. K., and Pickels, E. G., *J. Immunol.*, 1942, **45**, 273.

[3] Salk, J. E., *J. Immunol.*, 1944, **49**, 87.

TABLE II.
Relative Lack of Dependence of Hemagglutination Titer at a Given Temperature upon Prior Exposure at Another Temperature.

| Dilution | 0 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 | Control |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Temp. of exposure °C | | | | | | | | | | | |
| 0 | + | + | + | + | + | + | + | + | 0 | 0 | 0 |
| Room | + | + | sl+ | sl+ | 0 | 0 | 0 | 0 | | | 0 |
| 39 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | 0 |
| 45 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | 0 |
| Above tubes reshaken and placed at different temperatures: | | | | | | | | | | | |
| 45°C set at 0°C | + | + | + | + | + | + | + | sl+ | | | 0 |
| 39°C set at room | + | + | sl+ | 0 | 0 | 0 | 0 | 0 | | | 0 |
| Room set at 39°C | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | 0 |
| 0°C set at 45°C | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Sheep cells used. Agglutination read by "pattern" test.

TABLE III.
Effect of Washing Upon Agglutinated Red Cells.
Chicken cells: 38 × 10⁶ per tube.

| Dilution | | | 0* | 2 | 4 | 8 | 16 | 32 | Control |
|---|---|---|---|---|---|---|---|---|---|
| Temperature of | | | | | | | | | |
| First agglutination, °C | Washing, °C | Agglutination after washing, °C | Agglutination results | | | | | | |
| 10 | — | — | + | + | + | + | sl+ | 0 | 0 |
| | 45 | 10 | + | + | + | + | hemolyzed | hemolyzed | 0 |
| 45 | — | — | + | + | sl+ | sl+ | 0 | 0 | 0 |
| | 25 | 45 | + | + | + | + | 0 | 0 | 0 |
| Sheep cells: 131 × 10⁶ per tube. | | | | | | | | | |
| 10 | — | — | + | + | + | sl+ | 0 | 0 | 0 |
| | 45 | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 45 | — | — | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 25 | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 45 | kept at 45° | | 0 | 0 | 0 | 0 | 0 | 0 | |
| | then placed at 10° | | + | + | sl+ | 0 | 0 | 0 | 0 |

* Tube contains 25,000 mouse MLD of crystalline toxin per ml.

Results within each horizontal column are those obtained with a single set of tubes. A total volume of 2 ml was present per tube. Diluent and wash fluid was saline. Washing was done twice by centrifugation of the cells from 5 ml of fresh saline at the indicated temperature.

animals they represent cell types with extensive morphological differences in both structure and size. It was thought to be better to use concentrations of these cells with equivalent surface areas rather than equal numbers since the effects of the toxin are assumed to primarily involve surface phenomena or structures. The sheep cells were more sensitive than the chicken cells to the temperature variable. But with both types of cells the effect of temperature was found to be relatively independent of the previous temperature exposures of red cell-toxin mixtures. Data for 0°C about the same endpoint is recorded sheep cells given in Table II show that at whether the test is conducted initially at 0°C or the cell-toxin mixture is initially held at 45°C and then placed at 0°C. Exposures at the other 3 temperatures listed in Table II show the same characteristic.

An interesting difference between agglutinated sheep and chicken cells was in their behavior when washed free of the suspending toxic fluid (Table III). Once chicken cells have been agglutinated they tend to remain agglutinated irrespective of washing or the temperature of washing. With agglutinated sheep cells the effect of washing was more complex. Washing tended to reduce the persistence of agglutination but was conditioned by the temperature of washing. In one experiment a set of tubes of sheep cells which

TABLE IV.
Inability of Antitoxin to Redisperse Agglutinated Chicken Red Cells.

| Dilution of toxin | 0* | 2 | 4 | 8 | 16 | 32 | 64 | Control |
|---|---|---|---|---|---|---|---|---|
| Treatment | | | | | | | | |
| Set 1: Toxin + 0.2 ml antitoxin for 30 min. at 25°C before adding red cells. Readings overnight at 10°C | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Set 2: Toxin + 0.2 ml saline for 30 min. at 25°C before adding red cells. Reading overnight at 10°C | + | + | + | + | + | + | 0 | 0 |
| Set 2: Reshaken. 0.2 ml antitoxin added. Kept at 25°C 2 hr, then at 10°C overnight for agglutination reading. | + | + | + | + | sl+ | 0 | 0 | 0 |

Horse antitoxin used was commercially prepared. According to the manufacturer's statement of potency about 3 units of antitoxin would be present in the 0.2 ml added to each tube of the experimental set. Red cells per tube were $40 \times 10^6$ Agglutination was read by "pattern" on bottom of tube.

* 100,000 mouse MLD of toxin were present in the first tubes.

had been agglutinated at 0°C when washed at 45°C showed a complete loss of agglutination. A duplicate set washed at 5°C showed persistence of agglutination in the tubes originally containing the greatest amounts of toxin. There was only a one or two tube reduction in endpoint with the 2-fold dilution schedule employed.

Agglutinated sheep cells washed free of toxin at 45°C so that they were no longer agglutinable at 0°C remained capable of being reagglutinated by the addition of fresh toxin.

As already stated antitoxin was found to inhibit hemagglutination irrespective of the order in which the reagents were mixed initially. But it was of some interest to learn whether or not the addition of antitoxin could disperse cells which had been once agglutinated. Accordingly experiments to test this point were performed. The antitoxin proved relatively incapable of dispersing agglutinated chicken red cells. Typical data are given in Table IV. In still other experiments the agglutinated chicken cells remained agglutinated even when washed free of toxin with saline, and then resuspended in a quantity of antitoxin sufficient to inhibit agglutination if the reagents had been added together at the beginning of the trial. Experiments along similar lines with sheep cells were performed but are not recorded since results were irregular and inconclusive.

The observations discussed are preliminary to an effort to explain hemagglutination by the toxin, and to learn whether hemagglutination can be employed as a quantitative measure of toxin. They have been presented for the purpose of bringing the phenomenon into common knowledge. It is not possible at the moment to finally decide whether the agglutination is due to an enzymatic action by the toxin on the red cell, or to some purely physical effect of the toxin solutions that unsettles the balance of cohesive and repulsive forces normally operative at the red cell surface. Nor is it possible to conclude whether one type of mechanism is acting in the case of chicken cells and another for the sheep cells. But an additional explanation of the results exists which is probably not supported by the evidence at hand. Lamanna and Doak[4] have found that normal serum can be precipitated by botulinal toxin. If in the studies performed a contaminating layer of normal serum proteins was present at the cell's surface then the agglutination of the red cells might be more apparent than real. The reaction between toxin and the serum might well explain the results. Objections to the occurrence of this event are as follows: All the chicken and sheep red cells used were washed by centrifugation from saline or a buffer-saline mixture. The amount of residual serum proteins must be minimal. Guinea pig red cells when washed four times by centrifugation from mammalian Ringer's solution remained agglutinable by toxin. The addition of normal serum to a mixture of red cells and toxin did not affect the agglutination titre.

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[4] Lamanna, C., and Doak, B. W., *J. Immunol.*, 1948, **59**, 231.

Yet the large excess of serum in these cases might logically be expected to compete for the toxin and thus reduce the hemagglutination titre. If normal serum proteins were in some way "bound" at the red cell's surface antitoxin might be similarly bound. The addition of toxin to a red cell-antitoxin mixture should then be expected to result in agglutination. Actually a mixture of sheep cells and horse antitoxin incubated for an hour before addition of toxin remains unagglutinated. Finally, the presence of serum at the cell surface should show some, if slight, toxin adsorption if such serum were actually responsible for agglutination. An experiment was performed in which 460 millions of sheep red cells were mixed with only 200 mouse MLD of toxin at 10°C. Putnam *et al.*[5] have determined that the number of molecules per $LD_{50}$ is of the order of 2.1 x $10^7$. An MLD would contain less than twice as many molecules.[6] Thus in the mixture used there was only of the order of 18 toxin molecules per cell. The adsorption of only a few molecules per red cell would have resulted in a large percentage adsorption of toxin. Yet when the red cells were centrifuged down and the supernate titrated no significant reduction of toxic titre was found.

*Summary.* Type A botulinal toxin causes agglutination of chicken, guinea pig, rabbit, sheep and human red cells. The agglutination is not accompanied by evidence of adsorption of the toxin by the red cells. Temperature, particularly in the case of sheep cells, influences the hemagglutination titre. When washed free of toxin, agglutinated chicken cells tend to remain agglutinated while agglutinated sheep cells are more readily dispersed.

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[5] Putnam, F. W., Lamanna, C., and Sharp, D. G., *J. Biol. Chem.*, 1946, **165**, 735.

[6] Lamanna, C., McElroy, O. E., and Eklund, H. W., *Science*, 1946, **103**, 613.

### 16711

## Regression and Reabsorption of Mammary Tumors by Extracts of Degenerating Amphibian Skin.

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The chemotherapy of cancer has received and still receives considerable attention. Most work has been directed towards the examination of substances which inhibit growth as contrasted with those which are curative in the sense of bringing about the complete regression of the tumor. The works of Roffo[1] and of Boyland[2] were of especial interest to the writer and led to the experiments here described. Roffo first found that the injection of fresh extracts of striated muscle into rats implanted with a transferable fibro-sarcoma 10-19 days previously were without inhibitory or regressional effects. The same negative findings were recorded when tumorous tissue was tested *in vitro*. However, striking regression and disappearance of tumorous tissue was obtained when applying hydrolysates of striated muscles both *in vivo* and *in vitro*. Boyland was able to demonstrate less striking results on grafted sarcomata and spontaneous carcinomata in mice following oral administrations of acid extracts of ox heart muscle. Pronounced inhibition of tumor growth was obtained but no cases of complete regression.

Reasoning from a general hypothesis that degenerating tissues might, in certain cases, contain a substance or substances detrimental to the growth or persistence of tumorous tissue, experiments were planned involving

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[1] Roffo, A. H., *Biol. Inst. Med. Exp.* (Buenos Aires), 1938, **14**, 257.

[2] Boyland, Eric, *Biochem., J.*, 1941, **35**, 1283.

TABLE I.
Effect of Skin Extracts and Control Solutions on Mammary Tumors in Mice.

| Case No. | Animal No. | Extract or control solution | Dosage (cc) | No. of injections | Original size of tumor* (mm) | Size 2 days after last injection† (mm) |
|---|---|---|---|---|---|---|
| 1 | Cn 1 | Cntr. | 0.3 | 6 | 34 | 33/12 |
| 2 | Cn 2 | Cntr | 0.3 | 6 | 37 | 22/12 |
| 3 | Cn 3 | Cntr. | 0.3 | 6 | 40 | 29/12 |
| 4 | CF 1 | CS | 0.3 | 6 | 29 | 0/12 |
| 5 | CF 2 | CS | 0.3 | 6 | 33 | 4/12 |
| 6 | CF 3 | CS | 0.3 | 6 | 31 | 7/12 |
| 7 | CF 4 | CS | 0.3 | 6 | 30 | 0/12 |
| 8 | Cn 4 | Cntr. | 0.15 | 6 | 26 | 24/12 |
| 9 | Cn 5 | Cntr. | 0.15 | 6 | 24 | 14/12 |
| 10 | Cn 6 | Cntr. | 0.15 | 6 | 25 | 16/12 |
| 11 | CF 5 | CS | 0.15 | 6 | 22 | 7/12 |
| 12 | CF 6 | CS | 0.15 | 6 | 17 | 0/12 |
| 13 | CF 7 | CS | 0.15 | 6 | 24 | 0/12 |
| 14 | Cn 7 | Cntr. | 0.05 | 9 | 30 | 32/18 |
| 15 | Cn 8 | Cntr. | 0.05 | 9 | 21 | 17/18 |
| 16 | CF 8 | CS | 0.05 | 9 | 20 | 0/18 |
| 17 | CF 9 | CS | 0.05 | 9 | 29 | 14/18 |
| 18 | CF 10 | CS | 0.05 | 9 | 31 | 11/18 |
| | | | Subcutaneous Injections. | | | |
| 19 | Cn 9 | Cntr. | 0.3 | 9 | 35 | 40/18 |
| 20 | CF 11 | CS | 0.15 and 0.3‡ | 9 | 25 | 22/18 |
| 21 | CF 12 | CS | 0.15 and 0.3‡ | 9 | 29 | 22/18 |
| 22 | CF 13 | CS | 0.3 | 9 | 40 | 38/18 |

* Length plus breadth.
† The numerator = length plus breadth; denominator = number of days since first injection.
‡ Five injections of 0.15 cc followed by four of 0.3 cc.

the effects of extracts of degenerating amphibian tail muscle and skin on spontaneous tumors in mice.

*Materials and methods.* Extracts of normal and degenerating anuran larval tail muscle and skin were prepared. Preliminary tests showed that those of degenerating skin were most effective. These (Extract CS) were prepared as follows: *R. catesbeiana* larvae were selected in a metamorphic stage at which tail resorption was well underway. The tail integuments of approximately 60 larvae were stripped off and ground to a homogeneous consistency in a mortar. 25 cc of cold 5N HCl was added to 5 cc of the ground skin and the mixture allowed to stand, at room temperature, for 48 hours. The mixture was then filtered several times and the final filtrate neutralized against litmus with dry $NaHCO_3$. A small amount of sterile distilled water was now added to bring the volume up to 25 cc. The extract was then placed in a sterile bottle and refrigerated until ready to use. Neutral control solutions, for injection, were also prepared with 5N HCl and $NaHCO_3$.

CFl strain mice* of uniform age and showing definite surface indications of early spontaneous mammary tumor growth were utilized for injection purposes. The mice selected for injection were placed in individual cages and maintained under uniform diet conditions for at least a week prior to use in the tests. The selection of individuals for extract and control solution injection purposes was made on the basis of general healthiness of the animals and similarity in tumor size and location. Extracts and control solutions were injected in most cases directly into the tumors and in a few cases subcutaneously on the back. The injections were made every other day.

*Results.* The results are listed in Table I. They include only those animals which lived for more than 10 days following the last injection. Such mortality (27%) as occurred was distributed quite equally among extract and control injected and non-injected stock animals. The mortality was attributed entirely to extreme fluctuations in room temperature beyond control of the writer. The injected animals lived frequently for 30 or more days following the last injection. In certain individuals no new tumors appeared. In others one or two developed but never in close proximity to the injection site. For most cases involving tumor regression the effect was noted within 3 to 6 days, after which reduction in size progressed steadily.

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* The mice were supplied by Carworth Farms, New City, N. Y.

In 6 cases (not listed in Table I) injections were terminated when the tumors were reduced approximately 50%. In 4 of these, tumor regression continued, but at a lowered rate, while in the other 2 regression ceased shortly and the tumors enlarged subsequently. New tumors appeared in several of the cases listed in Table I at the same time the injected tumors were undergoing reduction in size. This was not true, however, of any of the subcutaneously injected animals.

A study of the results listed in Table I will indicate that tumors showed definite regression following injections of control solutions in doses of 0.3 and 0.15 cc while 0.05 cc injections were relatively ineffective. Extract injected tumors, however, evidenced much greater regression in rate and size in all dosages used and accounted for the five cases of complete resorption.

Histological studies were made at successive growth stages of normally developing CFl strain mammary tumors and also of tumors undergoing degeneration as the result of control solution and skin extract injections. The normal stock tumors showed the usual transformations from simple adenomatous to malignant carcinomatous types. Tumors injected several times with control solutions usually showed cystic development localized in one or more regions of the growth with large masses of carcinomatous tissue and some adenomatous in the remaining regions. In contrast, extract injected tumors after several injections evidenced larger and more numerous cysts accompanied by considerable amounts of adenomatous but very little carcinomatous tissue.

The subcutaneous injection of skin extract and control solution (Table I, cases 19-22) is of interest in that inhibition of tumor growth and slight regression in size is suggested. The number of cases is too small, however, to justify any definite conclusion.

*Summary*. Neutralized acid extracts of degenerating tail skin from involuting *R. catesbeiana* larvae were injected into rapidly growing spontaneous mammary tumors in mice. Marked regression of the tumors was obtained in all instances resulting in complete disappearance of all tumorous tissue in 5 out of 13 cases within eight to eighteen days after the initial injection.

The application of neutralized acid control solutions, similarly injected, resulted with one exception in slight to marked reduction in tumor size but never in complete resorption.

The tumors of one control and 3 extract animals, subcutaneously injected, showed only slight regression in size but are indicative in that growth was apparently inhibited.

The results indicate that a neutralized HCl acid extract of degenerating anuran tail skin contains some substance or substances possessing inhibitory and degenerative effects on spontaneous mammary tumors in mice beyond those exhibited by a neutralized acid control solution similarly injected.

## 16712

# Response of Normal and Malignant Lymphoid Tissue to Non Specific Tissue Damage.*

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In the evaluation of possible therapeutic agents in experimental lymphoma it has become apparent that drugs producing lymphoid tissue atrophy may do so either by direct action on the lymphocyte itself or an indirect action initiating an "alarm" reaction. Bass and Freeman[1] using 95% ethyl alcohol as a stimulous have shown that lymphoma 6C3HED responds in a manner similar to that of normal lymphoid tissue. That the alcohol itself was not a lympholytic agent was evident, as larger amounts of ethyl alcohol diluted to 19% concentration produced no lympholytic effects. Certain agents, such as nitrogen mustard and urethane, produce atrophy of lymphoid tissue through two possible mechanisms: (a) initiation of an alarm reaction[2,3] and (b) a direct action of the drug upon the lymphoid organs. The latter effect, which is the most important one, has been demonstrated by administration of these agents to adrenalectomized mice bearing lymphoid tumors.[4] It is, therefore, apparent that screening technics for new lympholytic agents must take into consideration both the direct and indirect effect upon lymphoid tissue. The present study was undertaken to establish further relationship of malignant tissue response to the administration of established stress stimuli. Those selected for the study were 40% glucose[5] and 4% formaldehyde.[6] Since it has been reported by Selye,[7] using the rat as his experimental animal, that fasting is either an alarming stimulus in itself or potentiates alarming stimuli, the effect of fasting in mice was also included in this study.

*Materials and methods.* The pure strain C3H male mice employed in this study were obtained from Roscoe B. Jackson Memorial Laboratories, Bar Harbor, Maine. The C3H $F_1$ hybrids of mixed sexes were bred in this laboratory. All animals were maintained on Purina dog chow and allowed free access to water at all times.

Transplanted lymphoma, 6C3HED, was employed in this investigation. A small particle of tumor tissue was implanted subcutaneously into the right axillary region of each animal by means of a 15 gauge needle. Procedures initiating alarming stimuli were instituted when tumors had become definitely measurable (seven days after implantation in pure strain C3H mice; seven to eleven days after implantation in C3H $F_1$ hybrid mice).

Intraperitoneal injections of 95% ethyl alcohol, U. S. P., were given to tumor animals in daily doses of 0.02 ml per mouse for 2 days. Animals used for spleen and thymus weight studies received 1.5 ml per kg at the same intervals. An aqueous solution of 40% glucose was administered intraperitoneally in doses of 150 ml per kg of body weight daily for 2 days. Subcutaneous injections of 4% formaldehyde were given twice daily for two days in doses of 5 ml per kg of body weight. Agents repeated within one day were given

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* This investigation was supported by a research grant from the National Cancer Institute of the National Institute of Health, U. S. Public Health Service.

[1] Bass, A. D., and Freeman, M. L. H., *Science*, 1948, **107**, 114.

[2] Ludewig, S., and Chanutin, A., *Endocrinol.*, 1946, **38**, 376.

[3] Karnofsky, D., Graef, I., and Smith, H. W., *Am. J. Path.*, 1948, **24**, 275.

[4] Bass, A. D., and Feigelson, M., *Cancer Research,* in press.

[5] Reiss, M., Macleod, L., Golla, Y., *J. Endocrinol.*, 1943, **3**, 292.

[6] Selye, H., *Brit. J. Exp. Path.*, 1936, **17**, 234.

[7] Selye, H., *J. Clin. Endocrinol.*, 1946, **6**, 117.

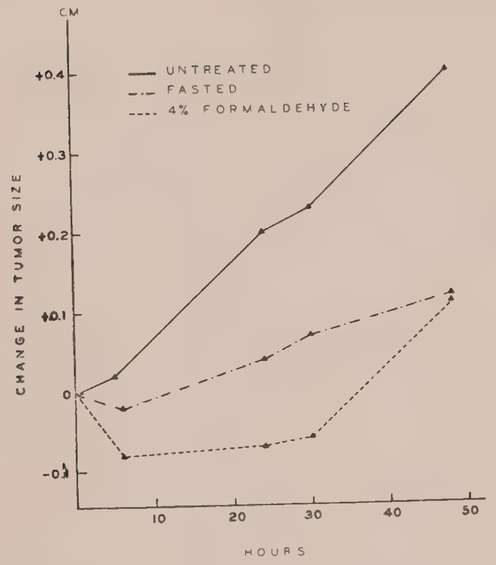

FIG. 1.

C3H hybrid mice were used. Number of animals employed: untreated 6, fasted 9, formaldehyde 10. Therapy was started at zero time.

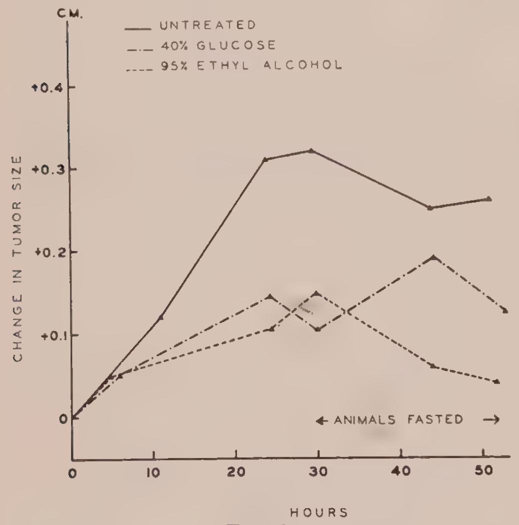

FIG. 2.

C3H mice were used. Number of animals employed: untreated 11, 95% alcohol 11, 40 glucose 12. Treatment was begun at zero time.

6 hours apart. Fasted animals were deprived of all food but were given water *ad libitum.* Tumor measurements were made twice daily. The longitudinal and transverse diameters of each tumor were measured with calipers and the mean dimension (one half of the length plus the width expressed in centimeters) was arbitrarily used as the tumor size. Animals used for organ weight studies were killed with ether 48 hours after therapy was initiated. Spleens and thymuses were carefully dissected out and weighed immediately on a Roller-Smith micro torsion balance.

*Results.* The effect of subcutaneous injection of 4% formaldehyde and of a 48 hour fast upon the tumor growth in C3H hybrid mice of mixed sexes is illustrated in Fig. I. It is clearly shown that the tumor growth curve of these treated animals is definitely altered from that of the untreated animals. Formaldehyde administration induced a definite regression whereas fasting induced only an inhibition of tumor growth. Fig. 2 shows the effect of two other alarm stimuli (95% ethyl alcohol administered intraperitoneally and 40% glucose administered intraperitoneally) on the change in tumor size in pure strain C3H male mice. Beginning 30 hours after the initial stimuli all animals were fasted for a period of 24 hours. Both stimuli (ethyl alcohol and glucose) are shown to inhibit tumor growth during the initial 30 hour period. Fasting under these conditions definitely potentiated the inhibiting effects of 95% ethyl alcohol and resulted in tumor regression in untreated animals. A less definite effect on the glucose treated animals was observed.

Table I demonstrates the effect of 95% ethyl alcohol, 4% formaldehyde, 40% glucose and a 48-hour fast on splenic and thymic wet weights in C3H $F_1$ hybrid mice of mixed sexes. Formaldehyde produced a statistically significant reduction in both spleen and thymus weights. Thymus weights of ethyl alcohol-treated animals and of fasted animals were significantly less than those of the untreated controls. Although all of the means of organ weights were not statistically significant, the mean spleen and thymus weights of all treated animals were lower than the untreated controls.

*Summary.* Further evidence has been added to indicate that ethyl alcohol is an alarming stimulus. We have shown that the non-specific stimuli, 4% formaldehyde and 40% glucose, in unadrenalectomized mice

TABLE I.
C3H Hybrids (Mixed Sexes) Sacrificed 48 Hours After Initial Therapy.

| | | | Organ weights, g per 100 g body wt | |
|---|---|---|---|---|
| Treatment | Dose schedule | No. animals | Mean spleen wt ± S.E. | Mean thymus wt ± S.E. |
| None | — | 15 | 1.348 ± 0.0802 | 0.153 ± 0.0194 |
| 95% ethyl alcohol | 1.5 ml/kg daily for 2 days | 7 | 1.050 ± 0.1259 | 0.051 ± 0.0101* |
| 4% formaldehyde | 5 ml/kg 2 times daily for 2 days | 9 | 0.605 ± 0.742* | 0.050 ± 0.0056* |
| 40% glucose | 15 ml/kg daily for 2 days | 10 | 1.055 ± 0.1187 | 0.112 ± 0.0152 |
| 48-hour fast | — | 9 | 1.138 ± 0.1031 | 0.107 ± 0.0147* |

* Upon statistical analysis weights are significantly smaller than untreated controls.

temporarily inhibit growth or produce temporary regression of the malignant lymphoid tumor 6C3HED. A similar effect is produced by a 48-hour fast. Fasting begun 30 hours after the initial stimulus gives an additional "lympholytic" effect.

It has been shown that stimuli which are known to produce an alarm reaction with resulting atrophy of the normal thymus and normal spleen produce a similar type of reaction in tissue composed of malignant lymphocytes.

## 16713

# Human Skin Grafted Upon the Chorioallantois of the Chick Embryo for Virus Cultivation.*

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(Introduced by Werner Henle.)

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Goodpasture, Anderson, and Douglas have described a technic for grafting human skin onto the chorioallantoic membrane of embryonated eggs.[1,2] They demonstrated that viruses such as herpes simplex and vaccinia, which multiply readily in the normal embryonated egg, would also grow in the grafted skin with the appearance of characteristic inclusion bodies in the epithelial cells. In addition, by using this technic they recorded an experiment in which they were able to grow herpes zoster on a single occasion.[3] They also indicated the value of such heterologous skin grafting in immunologic studies, by demonstrating that skin taken from chickens immune to fowl pox would lose its immunity after being grafted onto the chorioallantois.[4] It is assumed that the immune bodies are diluted out of the skin as it grows in the egg.

The obvious usefulness of vascularized, morphologically distinct human skin, actively growing in a sterile host that does not produce antibodies of its own, prompted the

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† Fellow in the Medical Sciences, National Research Council, during part of this study.

‡ Senior Fellow in Virus Research, National Research Council.

[1] Goodpasture, E. W., Douglas, B., and Anderson, K., *J. Exp. Med.*, 1938, **68**, 891.

[2] Goodpasture, E. W., and Anderson, K., *Am. J. Path.*, 1942, **18**, 563.

[3] Goodpasture, E. W., and Anderson, K., *Am. J. Path.*, 1944, **20**, 447.

[4] Goodpasture, E. W., and Anderson, K., *Arch. Path.*, 1940, **30**, 212.

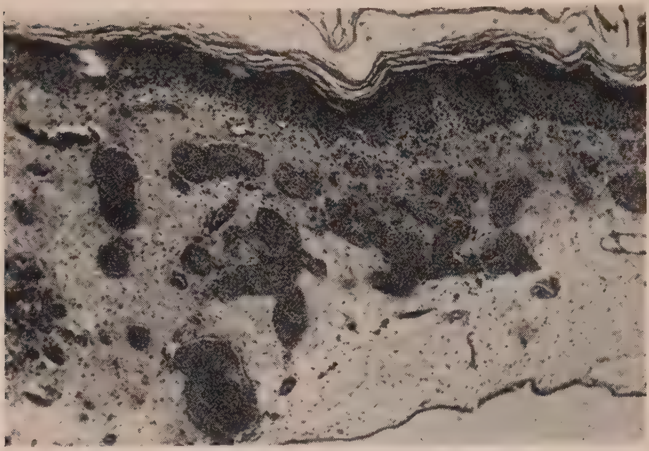

FIG. 1.
Human skin, 7 days after grafting onto chorioallantois. Large blood vessels contain nucleated erythrocytes, and are prominent in the denser human corium as well as the chick membrane. (H & E × 40).

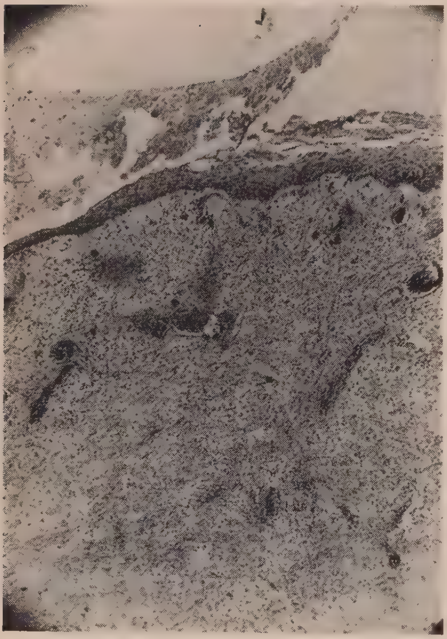

FIG. 2.
Human skin, after grafting onto 3 successive eggs for a total of 21 days. The epidermis remains distinctive. The dense collagen bundles of the corium have been separated by inflammatory cells of the chick. (H & E × 40).

study herein reported. This was to try to develop a relatively simple procedure whereby bacteria-free, growing skin was available which could be kept growing by serial transfer from egg to egg.

*Technic.* The human skin used in most of this study was obtained at circumcision of white and Negro children.§ The prepuce was cleaned preoperatively with soap and water and alcohol. Other antiseptics were avoided. The skin was used routinely within 2 hours of operation, but occasionally was stored in the ice box at 4°C for as long as 5 days before grafting. The prepuce was further cleaned of clotted blood and debris in the laboratory by bathing with the following solution: Physiological saline solution, phosphate buffered to pH 7.2, to which had been added gelatin to make 0.5% solution, and penicillin and streptomycin to make a final concentration of 500 units and 100 $\mu$g per ml, respectively. Previous studies in this laboratory[5] have indicated the antibacterial effectiveness and innocuous character of this solution. The skin was stretched, with sterile pins, on a sterile, solid cork board, epithelial surface down. With forceps and scalpel, the connective tissue was meticulously removed,

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§ Obtained through the courtesy of Dr. C. E. Koop and the Surgical Staff of the Children's Hospital of Philadelphia.

[5] Coriell, L. L., Blank, H., and Scott, T. F. McNair, to be published.

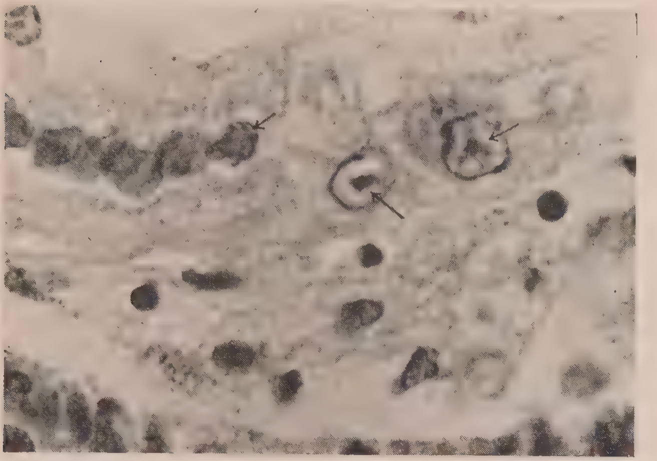

FIG. 3.
Epithelium of grafted human skin infected with herpes simplex 48 hours before fixation. This skin has been growing for 27 days (in 4 eggs) before inoculation with the virus. Intranuclear inclusion bodies in various stages of development are present. (H & E × 700).

leaving the epidermis and attached corium as thin as possible. Throughout this procedure, the antibiotic gelatin saline solution was used generously to prevent the tissue from drying. The thin sheet of skin was then cut into squares of the desired size. Ten-day-old embryonated hens' eggs were prepared by the "false air sac technic,"[6] and a window was made in the side of the egg overlying the false air sac by removing pieces of shell with the aid of fine forceps. A sterile, flat instrument, such as a forceps handle, was used to place the skin on the chorioallantoic membrane of the eggs, with the raw surface in even contact with the membrane. After grafting the skin, the shell defect was sealed with 2 layers of Scotch tape, and the eggs were incubated on their sides at 96°F.

The grafted skin was inoculated with virus-containing material 24 hours later, under the assumption that humoral antibodies from the donor of the skin would be largely removed after this interval on the egg.[2] The grafted skin was inoculated through a window cut in the Scotch tape with sterile scissors, after preliminary cleansing with 70% alcohol. Material to be inoculated was placed directly on the skin, which was then pricked with a vaccinating needle or with a 27-gauge hypodermic needle of the syringe used to deposit the inoculum onto the skin. More Scotch tape was used to seal the window again, and the eggs incubated for an additional period, varying from 1 to 7 days as the particular experiment required.

At the appropriate time, the skin was removed with attached chorioallantoic membrane. Material for histological study was placed on a small piece of absorbent paper with the membrane next to the paper, and the whole fixed in 5% acetic acid Zenker's fixative. Hematoxylin- and eosin-stained paraffin sections were prepared for routine study.

For passage to another egg, the skin was removed aseptically and placed in a sterile petri dish. With the aid of iris scissors and forceps, all of the attached chorioallantois was trimmed away, as was any additional connective tissue which had grown in the corium during the initial period on the egg. The skin was then transferred to the chorioallantois of another 10-day egg, prepared in the usual fashion. Such serial passages were usually made at 7-day intervals.

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[6] American Public Health Association, Diagnostic Procedures for Virus and Rickettsial Diseases, p. 243, 1948.

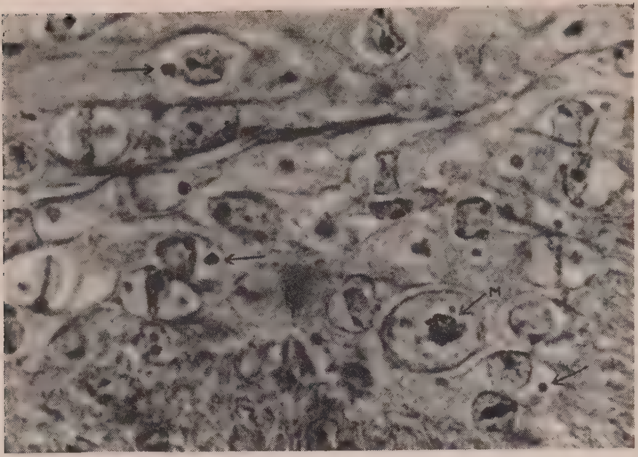

FIG. 4.
Epithelium of grafted human skin infected with vaccinia 72 hours before fixation. Numerous basophilic cytoplasmic inclusion bodies (Guarnieri) are present. A mitotic figure (M) can also be seen. (H & E × 450).

*Results.* The observations of Goodpasture and his group that human skin would grow quite readily on the chick chorioallantois were confirmed. Of 245 grafts only 13 were unsuccessful. Bacterial contamination was rare in our experience, although fungi such as *aspergillus* or *candida* occasionally grew out. A successful "take" was accomplished by active proliferation of the epidermis and the establishment of chick circulation in the human corium, as indicated by nucleated chick erythrocytes in the blood vessels of the corium (Fig. 1). This epidermal proliferation produced an increasingly thick, horny layer, which was desquamated after 2 or 3 weeks of continuous passage. If the epithelium failed to grow, the histologic picture was quite distinct. The epithelial cells assumed a light pink color with a small, pyknotic, dense nucleus, and there was none of the usual evidence of multiplication such as mitotic figures or increase in thickness. It was further demonstrated that the human skin could be passed serially at weekly intervals from egg to egg, and retain distinctive epithelium (Fig. 2). Of 124 regrafts, 7 failed to take.

By inoculating skin which had thus been kept alive for 4 weeks (27 days) with herpes simplex virus, and incubating for an additional 48 hours, it was possible to demonstrate the formation of characteristic intranuclear inclusion bodies in the epithelial cells of the skin graft. This was considered addi-

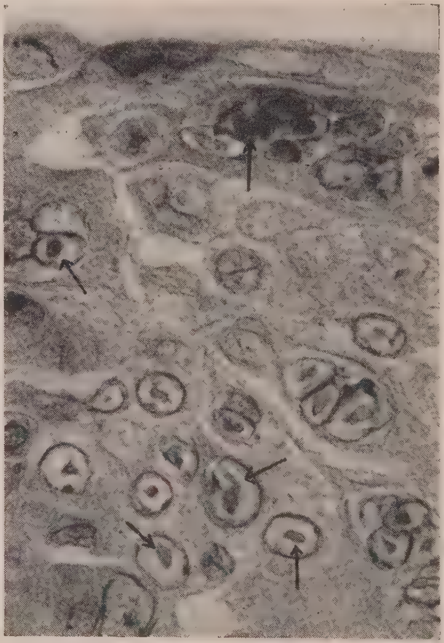

FIG. 5.
Epithelium of grafted human skin infected with herpes zoster 6 days before fixation. Almost every nucleus shows marginated chromatin and contains a characteristic inclusion body. (H & E × 450).

tional proof of the viability of the growing epidermis (Fig. 3). If the skin is inoculated with vaccinia virus, characteristic cytoplasmic basophilic inclusion bodies are formed in the epithelial cells (Fig. 4).

In addition, we were able to grow the virus of herpes zoster on one occasion (Fig. 5). This was done by inoculating skin which had been growing on the chorioallantois for 24 hours. The inoculum was untreated vesicle fluid taken from a patient with herpes zoster ophthalmicus of 48 hours' duration. The skin was removed after 6 days' additional incubation. The microscopic appearance of the infected area, including the presence of a large number of characteristic intranuclear inclusion bodies, confirms Goodpasture's observation in his single successful cultivation of the zoster virus.[3]

*Summary and Conclusions.* (1) Human skin may readily be grafted onto the chorioallantoic membrane of embryonated eggs. (2) The prepuce removed by usual surgical circumcision is a satisfactory source of adequate amounts of skin. (3) Penicillin and streptomycin obviate most bacterial contamination. (4) Grafted human skin may be passed serially from egg to egg at weekly intervals and remain viable. (5) Herpes simplex and vaccinia grow readily on grafted human skin, with the formation of characteristic inclusion bodies. (6) A successful inoculation with herpes zoster confirms the previous single successful cultivation of this virus outside of the human body.

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## 16714

# Atrio-Ventricular Anastomosis: An Additional Valve.

A. RAPPAPORT, J. F. MURRAY AND L. S. DAVIES. (Introduced by C. H. Best.)
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In vascular surgery during the last few years communications have been established experimentally and therapeutically between blood vessels. Little has been done in surgery of the heart involving the formation of anastomosis between the cavities of this organ. Schepelmann[1] and Dimitrieff[2] were the first to create artificial communications between the atria or the ventricles. Neither of these workers tried to produce a new opening that would join an atrium to a ventricle.

If such an anastomosis could be created with valvular properties it would be of value in pathological changes of the valves. In stenosis of the mitral valve, for example, the blood which has been dammed back in the atrium could stream through the new pathway into the left ventricle. In insufficiency, on the other hand, the anastomosis could serve as a safety valve guarding against the rising pressure in the atrium. These points, however, remain to be settled experimentally.

Dimitrieff[2], Cutler[3] and Souttar[4] have shown that various operations on the auricular appendage are well tolerated. The appendage also represents the shortest anatomical pathway for producing a connection between atrium and ventricle. Furthermore, by using it for the atrio-ventricular anastomosis, valvular requirements can be fulfilled. Because of its funnel shaped structure, it is most suitable for transformation into a valve and for implantation into the ventricle. In an anastomosis using the appendage, the blood will flow from a broad base to a narrow apex, whereas back flow through this structure would be difficult. The experiments to be described

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[1] Schepelmann, E., *Deutsche Z. f. Chir.*, 1912, **120**, 562.

[2] Dimitrieff, I. P., *Zentralbl. f. Chir.*, 1926, **53**, 715.

[3] Cutler, E. C., and Beck, C. S., *Arch. Surg.*, 1929, **18**, 403.

[4] Souttar, H. W., *Brit. Med. J.*, 1925, **2**, 603.

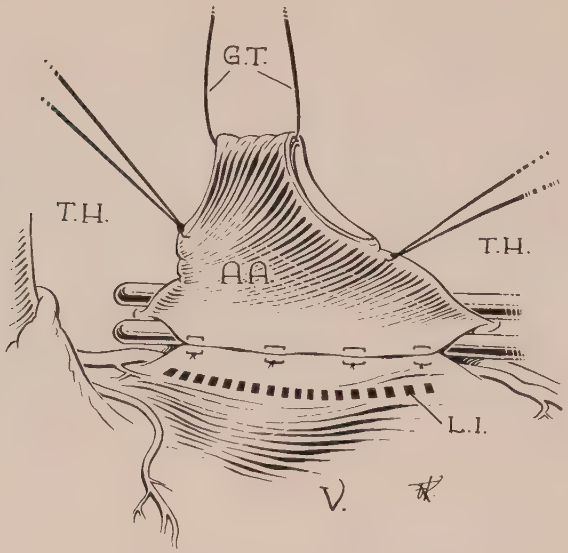

FIG. 1. Preparation of Auricular Appendage Showing Posterior Row of Sutures.
A.A. = Auricular appendage, posterior surface.
G.T. = "Guide thread."
L.I. = Line of ventricular incision.
T.H. = "Temporary hemostatic sutures."
V. = Ventricle.

were planned and initiated by the senior author (A.R.).

*Technic.* Dogs were anesthetized with intravenous nembutal and artificial respiration administered by positive pressure through an endotracheal tube. The animal was placed on the right side, a small pad under the right thorax providing the best exposure. The incision was usually made in the fourth intercostal space for the left auricle, the third intercostal space for the right. When the pleural cavity is opened a tampon soaked in 5% cocaine solution is used to paint the pericardium in order to block reflexes that might arise and disturb cardiac activity. Exposure is maintained by self-retaining rib spreaders and towels packing off the lungs. A longitudinal incision is made in the pericardium between two holding sutures which stretch and maintain the opening. The incision is carried up to the superior reflection of the pericardium. Cocaine solution (4%) is also introduced into the pericardial cavity and is spread over the epicardium by the heart contractions. The auricular appendage is gently grasped at its apex with tongue forceps, and a narrow slightly curved clamp is fixed as close as possible to its base. A row of interrupted mattress sutures, placed as close as possible to the clamp, joins the posterior wall of the appendage to the myocardium of the adjacent ventricle, just below the circumflex coronary vessels. Occasionally it is necessary to tie small branches of the coronary artery crossing this row of sutures. This does not cause any apparent damage as there is abundant coronary anastomosis in the dog heart (Moore[5]).

*Preparation of the Auricular Appendage.* The appendage can be opened on the anterior or posterior surfaces, by resection of the tip, or by bisecting it and everting the two resulting flaps. In this series longitudinal openings were cut along the borders of the appendage (Fig. 1). This creates two flaps which are joined at their tips, and therefore a valvular action can be expected. The adjacent walls are separated with fine curved forceps which are used to draw a paraffin-soaked thread through the two lateral incisions. This will be the "guide thread" (Fig. 1). The ends of this thread are held in a clamp.

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[5] Moore, R. A., *Am. Heart J.*, 1930, **5**, 743.

A suture is placed at the proximal limit of the opening on each side, tied into place (Fig. 1) and both free ends are threaded through a No. 6 curved round needle. They will be "temporary hemostatic sutures." The needles are clamped in needle holders and stuck in the drape sheets, ready for use.

*Opening the Ventricle.* A transverse incision is made in the anterior ventricular wall 5 mm below the posterior row of sutures already placed (Fig. 1). A broad scalpel or a punch which will produce a hole 15 mm long and 5 mm wide is used. After this has been pushed through the ventricular muscle a fine, curved Reverdin needle is introduced into the heart cavity about 3 cm below the ventricle opening. When it contacts the scalpel or punch the latter is pulled out, the Reverdin needle follows in its wake and the tip appears in the incision. The blood then spurts. The "guide thread" is threaded in the Reverdin needle and the latter is pulled out, quickly drawing the appendage into the incision and ventricular cavity. Hemorrhage is thereby diminished. While an assistant arrests further hemorrhage with direct digital pressure the "temporary hemostatic sutures", held in readiness, are guided into the ventricular cavity at the right and left corners of the incision. These are directed from within the cavity through the entire thickness of the ventricular wall (Fig. 2). Hemorrhage is completely arrested when these are drawn together and held by a single knot fixed in a hemostat.

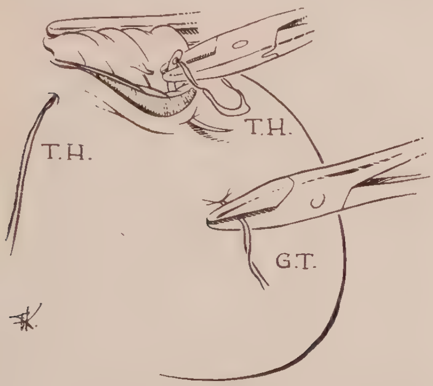

FIG. 2. Implantation of Auricular Appendage. Placing the "temporary hemostatic sutures." The auricular appendage is being held in position by a forceps on the "guide thread."

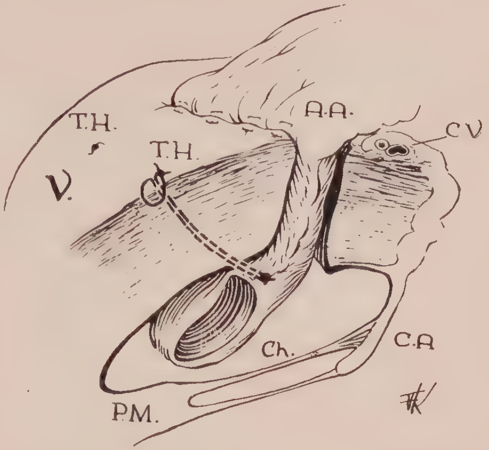

FIG. 3. Longitudinal Section of Anterior Ventricular Wall. Embedded Appendage "in situ."

C.A. = Anterior cusp.
Ch. = Chordae tendineae.
P.M. = Papillary muscle.
C.V. = Coronary vessels.
T.H. = "Temporary hemostatic sutures"—anchored.

The anastomosis is completed by interrupted mattress sutures uniting the anterior wall of the appendage to the edge of the ventricular incision. The single knot of the "temporary hemostatic suture" is opened and each double thread is permanently fixed where it emerges. These anchor the edges of the auricular appendage to the endocardial margin of the incision and hold it deeply implanted in the ventricular cavity (Fig. 3). The guide thread is pulled out after cutting one end short. The auricular appendage, now mobile, lies between the chordae tendineae of the anterior valve and the anterior ventricular wall (Fig. 3). If the appendage is too short to be implanted into the ventricle, it can be embedded in an incision made closer to its base, behind the coronary vessels. These are retracted after careful dissection. The ventricle is opened by an obliquely placed incision on the upper limit of the ventricular myocardium, just below the A-V ring. The appendage is then also lodged between the chordae tendineae and anterior wall of the ventricle.

The pericardium is left open, 100,000 units of penicillin are put into the thoracic cavity and the chest closed in the usual manner. The post-operative course is uneventful.

*Observation.* No regurgitation through the anastomosis was ever observed. As long as the clamp at the base of the auricular appendage remains closed, no ventricular pulsations in the appendage and no hemorrhage are seen. After the clamp is opened, blood from the atrium streams through the anastomosis and pulsates in rhythm with the atrium. Bleeding may now occur around stitch openings accidentally torn in the thin appendage wall, but it can easily be controlled.

This operation has been performed on 13 dogs of various size. One death occurred during the operation. The reason for the relatively good survival incidence could be found in the fact that the heart during the whole period of surgical intervention remains fully active and its muscle supplied with fresh oxygenated blood. No attempt is made to operate in a bloodless field; rather the main consideration is given to reducing to a matter of seconds that phase of the operation when the ventricle is opened. This is achieved by means of the technic described above. The blood loss varies between 50 and 150 cc. After all, this technic deals with quickly taking care of a wound which has been produced in the heart before our very eyes, and that problem has long been solved in surgery.

The authors are indebted to Professor C. H. Best, who has shown continuous interest and provided facilities for these experiments. They are also grateful to Dr. J. Markowitz for his helpful advice and encouragement.

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## 16715

# Experimental Hatching of the Eggs of *Ascaris suum*.*

THOMAS D. PITTS. (Introduced by Gordon H. Ball.)
*From the Department of Zoology, University of California, Los Angeles.*

Yoshida and Toyoda[1] reported the *in vitro* hatching of the eggs of *Ascaris suum* by direct incubation of the eggs for 5 days in a variety of media. Fenwick[2] did not secure hatching in several of the media reported by Yoshida and Toyoda,[1] but he consistently produced hatching in dilutions of Milton solution, a commercial sodium hypochlorite preparation. Fenwick[3] secured living larvae by treating infective eggs with a 1:15 dilution of Milton solution for 12 hours at 38°C, expressing the larvae between a slide and coverslip, and then washing the liberated larvae with an experimental saline. Pick[4] reported the hatching of the eggs of *A. megalocephala* in a Ringer-horse serum solution 34 days after the liquid had evaporated, but during which time the residue had been kept moist. Pick[4] further observed spontaneous hatching among eggs 21 days after transferring them from a Ringer-horse serum solution to a glucose agar medium.

As a preliminary step in the study of the culture requirements of the larvae of *A. suum*, it was necessary to develop a method of hatching *Ascaris* eggs that would provide large numbers of living larvae in a short time.

*Materials and methods.* Fertile eggs were secured from the mature female *Ascaris* worms from the small intestines of hogs. The eggs from individual worms were incubated at 23° to 25°C in 40 ml of 2% formalin in separate 250 ml cotton-stoppered Ehrlenmeyer flasks.

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* The early phase of the work was accomplished through a research fellowship awarded by the Zoological Society of San Diego under a grant from the Ellen Browning Scripps Foundation.

[1] Yoshida, S., and Toyoda, K., *Livro Jubilar do Professor Lauro Travassos*, 1938, 569.

[2] Fenwick, D. W., *J. Helminth.*, 1939, **17**, 69.

[3] Fenwick, D. W., *J. Helminth.*, 1939, **17**, 211.

[4] Pick, F., *Bull. de la Societe de Pathologie exotique*, 1948, **41**, 208.

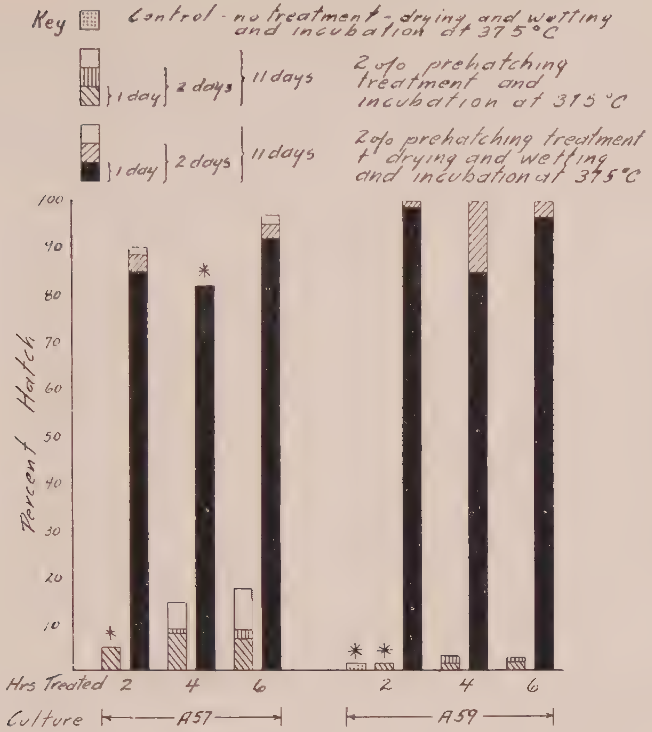

FIG. 1.

Bar graphs showing hatching results following treatment of *Ascaris* eggs in 2% "Clorox"-2% sodium hydroxide solution and subsequent drying, wetting and incubation.

* Per cent hatch did not increase after 1st day.

Under these conditions all eggs became embryonated in 24 days in most flasks. Cultures selected for hatching trials were at least 30 days old to allow for molting (Alicata[5]), had no eggs in stages earlier than the embryo, and showed at least 50% of the embryos motile.

Eggs to be used in hatching tests were treated at 37.5°C in a solution composed of 2% of the commercial sodium hypochlorite (5.25%) preparation "Clorox" and 2% sodium hydroxide. They were then washed five times in physiological saline. Eggs from individual worms were handled separately.

In the hatching tests 3 different methods were used:

1. Treated and washed eggs, in a few drops of physiological saline in shallow watch glasses, were incubated in moist chambers.

2. Suspensions of treated and washed eggs in shallow watch glasses were rapidly evaporated at 37.5°C with the aid of a fan. When salt crystals appeared throughout the suspension in each watch glass, the residue was rewet with saline and incubated in a moist chamber at 37.5°C.

3. Treated and washed eggs were centrifuged in physiological saline and the liberated larvae were available immediately after centrifuging.

*Results.* Some trials by method 1 resulted in all eggs hatching in 4 weeks of incubation at 40°C. However, most early-hatched larvae did not survive the length of time required to produce a high hatch.

By method 2 the liberation of larvae was more rapid, resulting in 100% hatches in 2 days in some trials. (Fig. 1). Up to 83.5% of the liberated larvae were alive in the hatching media at the end of 24 hours.

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[5] Alicata, J. E., U.S.D.A., Tech. Bull. No. 489, 1935, 44.

TABLE I.
Results of Hatching Eggs of *A. suum* by 24 Hour Treatments in 2% "Clorox"-2% Sodium Hydroxide Solutions and Subsequent Centrifugation at 3000 R.P.M. for 10 Minutes. Starting Centrif. Temp. 40°C.

| Orig. No. | Culture age (days) | % hatch |
|---|---|---|
| A76 | 37 | 77.1 |
| A77 | 37 | 74.7 |
| A78 | 37 | 65.8 |
| A64 | 57 | 98.6 |
| A61 | 57 | 99.1 |
| A61 | 60 | 87.8 |
| A76 | 67 | 74.2 |
| A78 | 67 | 77.4 |
| A77 | 104 | 70.5 |
| A79 | 104 | 58.1 |

Method 3 produced hatching as high as 99.1%, and up to 87% of the liberated larvae were motile 24 hours after hatching. A treatment time of 24 hours in a 2% "Clorox"-2% sodium hydroxide solution followed by centrifugation at 3000 R.P.M.† for 10 minutes, gave consistently high hatches. (Table I). At the end of the 10-minute centrifuge period the temperature in the hatching tubes had dropped from 40°C to approximately 25°C.

*Discussion.* In a "Clorox"-sodium hydroxide solution the shells of *Ascaris* eggs are dissolved, so that after 24 hours many embryos are surrounded by thin elastic shell membranes only. At this stage motile embryos may produce prominent bulges in these elastic envelopes and may be liberated by their own activities. Later, a complete dissolving of the shells including the membranes may release the embryos in the treatment solution. The shells of eggs from the same worm do not dissolve at a uniform rate, and cultures of eggs from different worms show differences in the average speed at which the shells dissolve.

The hatching of treated eggs incubated in saline, as in method 1, appeared to be due to the penetration of the shell membrane by the active larvae and to some extent the result of osmotic pressure.

Hatching by drying and wetting treated eggs probably resulted from physical stresses, set up in the chitinous shells when the eggs were dried, which caused the shells to fracture when rewet, rupturing the shell membranes beneath and liberating the larvae.

The pressure of membrane covered eggs against each other and the pressure of larvae against the sides of their membranous envelopes is a probable explanation of the hatching of treated eggs during centrifugation. Significant increases in hatching that appeared when the starting centrifuge temperatures were increased from 20° to 40°C were probably due to the liberation of larvae by their own efforts, due to greatly stimulated activity of the embryos at the higher temperature.

The motility of the larvae after hatching varied with the treatment time and with hatching conditions.

A study of photomicrographs in the report of Yoshida and Toyoda[1] revealed that the eggs pictured had no typical mammillated layer, but were essentially the same in appearance as those treated in "Clorox"-sodium hydroxide solution. The eggs were photographed in a 0.05% peptone solution, which in the present work was found to have no effect on the shells. These workers caution that all things used in hatching must be disinfected, and, although this phase of their technic is not described, it is possible that they used a sodium hypochlorite disinfectant, or some other chemical which had the same effect on the egg shells. If *Ascaris* eggs were permitted to remain in contact with such a disinfectant, the shells would be dissolved as shown in the photomicrographs and would probably have hatched in a variety of media as reported.

*Summary.* 1. Infective *Ascaris* eggs treated in "Clorox"-sodium hydroxide solution, hatched and produced viable larvae when they were subsequently:

(a) incubated in physiological saline at 37.5° and 40°C. Hatching occurred up to 100% in 4 weeks.

(b) dried, rewet and incubated in saline at 37.5°C. Hatching occurred up to 100% in 2 days.

(c) centrifuged at speeds from 2000 to 3000 R.P.M. Hatching occurred up to 99.1%

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† 1958 gravities.

during 10 minutes of centrifugation at 3000 R.P.M. with a starting temperature of 40°C.

2. Batches of eggs from different worms showed different hatching characteristics depending on the treatment strength and time, and influenced by subsequent procedure.

## 16716

# Effect of Insulin, Adrenal Cortical Hormones, Salt and dl-Alanine on Carbohydrate Metabolism in Scurvy.*

Hazel C. Murray. (Introduced by A. F. Morgan.)

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Previous work[1,2] has shown that scorbutic guinea pigs display a disturbance of carbohydrate metabolism resulting in lowered glycogen stores in liver and body. Murray and Morgan[1] demonstrated that when glucose was fed the scorbutic animal failed to absorb it as readily as the normal animal. The blood sugar after this procedure was also abnormally high. Sigal and King[3] had previously demonstrated a lowered glucose tolerance in scorbutic animals.

Banerjee[4] has demonstrated in scorbutic guinea pigs an increase in adrenaline production. He attributed the disturbance in carbohydrate metabolism to an imbalance between adrenaline and insulin since the insulin content of the pancreas appeared to be markedly decreased in the scorbutic animal.[5]

Involvement of the adrenal gland in the changes incident to ascorbic acid deficiency has been suggested. Marked hypertrophy of this gland has been shown to accompany this deficiency. Sayers, Sayers, Liang and Long[6] found that the injection of adrenotrophic hormone lowered both the cholesterol and ascorbic acid content of the adrenals of rats and guinea pigs suggesting that these 2 compounds are involved in the synthesis of cortical hormones. This hormone also greatly increased the deposition of liver glycogen in both species.

Intestinal absorption has been shown to be impaired in adrenalectomized rats[7,8] and the improvement of this condition demonstrated by adding sodium chloride to the drinking water. There was a possibility that the decrease in absorption in the scorbutic animal could be improved by adding this compound to the drinking water.

MacKay and co-workers[9,10] have shown that dl-alanine when fed to fasting mice or rats results in the formation of a considerable amount of hepatic glycogen. The possibility existed therefore, that the scorbutic animal could convert compounds of 3 C atoms to glycogen normally, and this might be some index of the adrenal cortical function.

This study was undertaken to determine the effect of insulin, adrenal cortical extract and additions of sodium chloride in the drinking water on glucose absorption, blood sugar levels, glycogen content of liver and body in scorbutic guinea pigs. The effect of adrenaline on blood sugar, body and liver glycogen and the conversion of dl-alanine to glycogen were also investigated. The diverse roles of the adrenal and pancreatic hormones in ascorbic acid deficiency might thus be indicated.

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* Published as Scientific Paper No. 786, College of Agriculture and Agricultural Experiment Stations, Institute of Agricultural Sciences, State College of Washington, Pullman, Wash.

[1] Murray, H. C., and Morgan, A. F., *J. Biol. Chem.*, 1946, **163**, 401.

[2] Hamne, B., *Acta Paediat.*, 1941, **28** Suppl.

[3] Sigal, A., and King, C. G., *J. Biol. Chem.*, 1936, **116**, 489.

[4] Banerjee, S., *J. Biol. Chem.*, 1945, **159**, 327.

[5] Banerjee, S., and Ghosh, N. C., *J. Biol. Chem.*, 1947, **168**, 207.

[6] Sayers, G., Sayers, M. A., Liang, T. Y., and Long, C. N. H., *Endocrinology*, 1946, **38**, 1.

[7] Clark, W. G., and MacKay, E. M., *Am. J. Physiol.*, 1942, **137**, 104.

[8] Anderson, E., Joseph, M., and Herring, V. V., Proc. Soc. Exp. Biol. and Med., 1940, **44**, 477.

[9] MacKay, E. M., Wick, A. N., and Carne, H. O., *J. Biol. Chem.*, 1940, **132**, 613.

[10] MacKay, E. M., Wick, A. N., and Barnum, C. P., *J. Biol. Chem.*, 1941, **137**, 183.

*Experimental.* Young guinea pigs (550-600 g) of both sexes were used. They were paired as to sex and weight. Animals were pair-fed, the normal animal of each pair receiving the amount of basal diet that the deficient member had consumed the previous day. The basal diet used was a commercial rabbit food.[†] In addition to the diet all animals received a carotene in oil supplement sufficient to supply 1 mg of carotene daily. The normal animals were given a solution containing 10 mg of ascorbic acid 3 times weekly. All the animals were kept at a temperature of 75-80°F.

The animals kept on this diet without ascorbic acid supplements exhibited a decreased appetite in 19-24 days. At this stage scurvy was present as evidenced by extensive hemorrhages of the fascia of the musculature particularly of the legs. A rapid loss of weight accompanied the loss of appetite. A few days after inanition became apparent in the deficient member of the pair, the carbohydrate technic of Cori and Cori[11] was applied to both animals. They were allowed to fast for 24 hours and were then given a solution containing 2.5 g of glucose orally. One hour later each member of the pair was given an injection of 20 units of insulin intraperitoneally and sacrificed 6 hours later by the use of sodium amytal.

Animals used for the experiments with adrenal cortical extract were treated similarly to those in the insulin experiment with the exception that the deficient animal only received an intraperitoneal injection of ½ ml of adrenal cortical hormone[‡] at hourly intervals 7 times in accordance with the assay procedure of Reinecke and Kendall.[12]

Animals used for the investigation of the effect of sodium chloride were treated as were the deficient animals in other groups except that a 0.5% solution of this compound was given for drinking water during the depletion period.

The effect of adrenaline was studied on animals prepared as were those in the above groups. After a 24 hour fast both the normal and deficient member of the group received an intraperitoneal injection of 0.15 ml of 1 to 1000 saline solution of adrenaline. They were sacrificed 3 hours later; blood sugar, liver and carcass glycogen determined.

In the investigation involving dl-alanine the deficient and normal animals were treated as were those in other groups. After a 24 hour fast a solution containing 1.4 g of dl-alanine was fed orally. Animals were sacrificed 12 hours later; liver and body glycogen determined.

The analytical procedure and methods as outlined by Murray and Morgan[1] were followed in all experiments.

*Results and Discussion.* The results of the analytical work are shown in Table I.

The injection of insulin failed to facilitate the storage of glycogen in the deficient animal; intestinal absorption was still impaired and blood sugar levels were high. Both the normal and deficient animals given insulin tended to show lower liver glycogen stores than animals not so treated; carcass glycogen did not seem to be affected. Cori and Cori[11] using the same technic with normal rats also found substantially lower amounts of liver glycogen in animals given insulin but body glycogen was comparable to that in the controls. Stetten and Klein[13] applying this same treatment to rats likewise found low liver glycogen levels, but large increases in carcass glycogen over

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[†] Purina rabbit pellets.

[11] Cori, C. F., and Cori, G. T., *J. Biol. Chem.*, 1926, **70**, 557.

[‡] Whole extract of the adrenal cortex—75 g/ml donated by Dr. E. C. Kendall, Mayo Clinic, Rochester, Minn.

[12] Reinecke, R. M., and Kendall, E. C., *Endocrinology*, 1942, **31**, 573.

[13] Stetten, D., Jr., and Klein, B., *J. Biol. Chem.*, 1945, **159**, 593.

TABLE I.
Effect of Insulin, Adrenal Cortical Extract, Adrenaline, Sodium Chloride and dl-Alanine on Carbohydrate Metabolism in Scorbutic and Normal Guinea Pigs.

| Treatment | No. of animals | Status | Glucose in intestinal tract, mg | Blood sugar mg, % | Liver glycogen % | Body glycogen % |
|---|---|---|---|---|---|---|
| None | 9* | Normal | 58 ± 20† | 92 ± 6† | 4.07 ± 0.20† | .528 ± 0.14† |
| | 9 | Deficient | 166 ± 41 | 162 ± 20 | 2.66 ± 0.33 | .439 ± 0.19 |
| | | | t = 2.34‡ | t = 3.33§ | t = 3.71§ | t = 3.90§ |
| Insulin 20 units | 8 | Normal | 17 ± 6 | 54 ± 7 | 1.70 ± 0.15 | .353 ± 0.03 |
| | 8 | Deficient | 157 ± 53 | 143 ± 48 | 0.52 ± 0.19 | .307 ± 0.08 |
| | | | t = 2.64‡ | t = 1.85 | t = 4.10§ | t = 0.52 |
| Adrenal cortical extr., 3.5 ml | 6 | Normal | 8 ± 4 | 135 ± 9 | 3.18 ± 0.38 | .372 ± 0.03 |
| | 6 | Deficient | 132 ± 60 | 230 ± 59 | 1.85 ± 0.34 | .324 ± 0.02 |
| | | | t = 2.07 | t = 1.61 | t = 2.60‡ | t = 1.33 |
| Adrenaline 0.15 ml 1-1000 | 6 | Normal | | 185 ± 9 | 0.92 ± 0.11 | .183 ± 0.02 |
| | 6 | Deficient | | 260 ± 30 | 0.62 ± 0.11 | .133 ± 0.02 |
| | | | | t = 2.40 | t = 2.0 | t = 1.80 |
| Sodium chloride | 8 | Deficient given NaCl | 138 ± 42 | 225 ± 49 | 1.34 ± 0.18 | .321 ± 0.03 |
| 0.5% in drinking water | 4 | Deficient no NaCl | 174 ± 50<br>t = 0.55 | 293 ± 106<br>t = 0.57 | 1.50 ± 0.29<br>t = 0.47 | .285 ± 0.02<br>t = 1.0 |
| dl Alanine 1.4 g | 8 | Normal | | | 0.79 ± 0.17 | .309 ± 0.02 |
| | 8 | Deficient | | | 0.05 ± 0.02 | .242 ± 0.03 |
| | | | | | t = 4.0§ | t = 1.60 |

* Figures taken from Table I, Murray, H. C., and Morgan, A. F., *J. Biol. Chem.*, 1946, **163**, 401.
† Standard error.
‡ Indicates significance at the 5% level.
§ Indicates significance at the 1% level.
Snedecor, G. W., Statistical Methods, Collegiate Press, Inc., Ames, Iowa, 1938.

fasting levels about 7-fold after feeding glucose and 3-fold after lactate.

Respiration trials run after feeding 2.5 g of glucose to 5 animals fasted 24 hours and injected with 4 units of insulin resulted in an average figure of 0.42 cc of oxygen per square centimeter of body surface per hour. When this same procedure was carried out on the same animals depleted of their ascorbic acid supply until scurvy appeared a figure of 0.43 cc was secured. The lower glycogen stores in the deficient animals given insulin can not then be due to increased oxidation.

Injections of adrenal cortical extract failed to improve absorption or promote glycogen storage. Kendall[14] noted that scorbutic animals given this extract survived longer than those not so treated but this did not cure the scurvy as the pathological lesions at death were the same whether animals received the treatment or not. Ratsimamanga[15] reported that scurvy produced an adrenal cortical deficiency and that injections of this hormone prolonged the survival period 1 to 3 weeks.

The addition of sodium chloride to the drinking water failed to improve any aspect of carbohydrate metabolism investigated in this study.

Adrenaline injections produced high blood sugar in both members of the pair; the level in the deficient animal was higher than that in the normal one. Lower glycogen levels were also found in the deficient animal, but the differences between the two groups were of no significance. Cori and Cori[16] observed that when rats were given adrenaline in the post absorptive state high blood sugar resulted, carcass glycogen was lowered and some increase in liver glycogen occurred.

The deficient animal was apparently not as competent in converting dl-alanine to liver glycogen as was the normal.

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[14] Kendall, E. C., Mayo Clinic, Rochester, Minn., private communication.

[15] Ratsimamanga, A. R., *Compt. rendu. Soc. de Biol.*, 1944, **138**, 19.

[16] Cori, C. F., and Cori, G. T., *J. Biol. Chem.*, 1928, **78**, lxii.

*Summary.* The injection of insulin or adrenal cortical extract in guinea pigs deficient in ascorbic acid did not materially alter the disturbance in carbohydrate metabolism characteristic of scorbutic animals; glycogen levels were still low, intestinal absorption impaired and blood sugar levels high after feeding glucose. Oxygen consumption of animals fed glucose then injected with insulin before and after depletion was unchanged. The addition of sodium chloride to the drinking water of deficient animals failed to improve any aspect of carbohydrate metabolism studied. Adrenaline given to both members of the pair produced higher blood sugar levels in the normal than in deficient animals and slightly higher levels of liver glycogen. Normal animals demonstrated much greater ability to convert dl-alanine to liver glycogen than did the deficient.

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## 16717

## Secretion in Cow's Milk of Intravenously Injected Radioactive Phosphorus $P^{32}$.*

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*From the University of California College of Agriculture, Davis, Calif.*

Since phosphorus plays a major role in intermediary metabolism, especially in biological energy transformations, the measurement of phosphorus transfer and turnover rates in domestic animals is important in research on the basic factors of food utilization.

This report deals with the rate at which intravenously injected $P^{32}$ is secreted in the milk, and its concentration in milk, as a function of time. These data are of particular interest for planning tracer experiments with large animals and for appraising cows as producers of labeled compounds, such as casein, for other trials. Comparing the secretion rate and concentration in milk of $P^{32}$ with that of $Sr^{89}$, mentioned earlier by Erf and Pecher,[1] leads to the tentative conclusion that the phosphorus stored in the body is more mobile than the calcium.

The excretion of $P^{32}$ in feces and urine, and specific activities in blood and other body constituents will be discussed in later papers in connection with the results of additional work still under way.

*Method.* Two lactating Jersey cows were used for the two trials on phosphate secretion reported in this paper. The same cows were, and still are, used in other trials with $P^{32}$. To simplify later comparisons, the cows will be identified by their herd number, and the trials by Roman figures.

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*This paper is the first report on a cooperative project, "Metabolic Research with Isotopes," started two years ago on the Davis campus among members of the Divisions of Chemistry and Animal Husbandry, and now including workers of other divisions. The authors take this opportunity to thank the colleagues who were instrumental for a promising start of this tracer work. Dr. H. Reiber of the Division of Chemistry provided us with the first sample of $P^{32}$. He also helped us with the analytical work during our first trial, as did Dr. J. K. Loosli, on sabbatical leave from Cornell University. Drs. M. E. Gardner and Charles G. Patten of the Division of Mathematics and Physics made many measurements of radioactivity for us and trained us in the use of the Geiger counter borrowed from Dr. W. B. Hewitt, Division of Plant Pathology. The generous help and advice of members of the Department of Medical Physics at Berkeley, especially Dr. H. Jones, has been most encouraging. Last but not least, we are glad to acknowledge the faithful and ever-ready help of Mr. Th. Chernikoff, technician in the Division of Animal Husbandry.

† At present at the Department of Medical Physics, Berkeley.

[1] Erf, L. A., and Pecher, Ch., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 762.

Cow 798 was 7 years old, weighed 400 kg, and was in the 5th month of lactation when, for the trial III, she was injected intravenously with 50 ml of a phosphate solution isotonic with blood and containing 15 millicuries of $P^{32}$. The same amount of a similar solution, but containing 30 millicuries of $P^{32}$, was injected into cow 890 which was 3 years old, weighed 370 kg, and had calved 5½ months before the trial started. Cow 798 produced daily 9.5 liters of milk; cow 890 8.7 liters. These production rates are means for the second to the seventh day after injection. The radioactive solution was injected into the jugular vein through plastic tubes otherwise used to insulate wires and known to electricians as "spaghetti". This method, which will be described in detail later, causes a minimum of excitement, and provides therefore a good chance that no "hot" solution is spilled or leaks into the tissues around the vein. The cows were milked just before injection, at 1, 2, 6 and 12 hours after injection, and later twice a day. The radioactivity of the milk was measured in 0.1 ml samples pipetted on 1″ diameter disks of lens cleaning paper, dried on 1¼″ diameter aluminum disks and then fixed with a spray of an alcoholic shellac dispersion.

*Results.* The concentration of $P^{32}$ in milk reached a maximum during the period of 3 to 8 hours after injection. This maximum amounted to 1.21% of the injected dose per liter milk of cow 798 in Trial III and to 1.24% per liter milk of cow 890 in Trial IV.

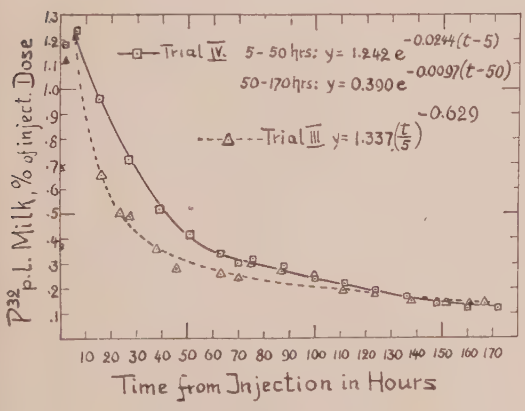

FIG. 1.
Radioactivity in milk.

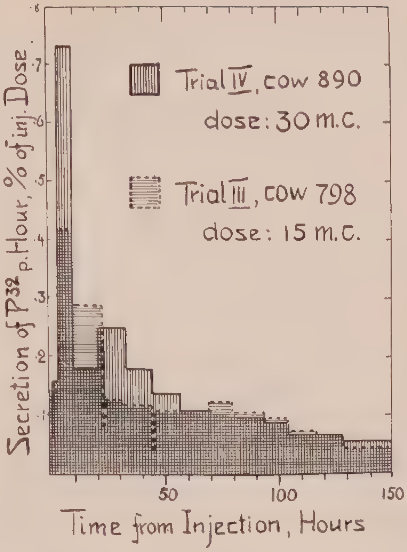

FIG. 2.
$P^{32}$ secretion.

Fig. 1 shows the $P^{32}$ concentration in milk as a function of time from injection.

The relative rates of secretion of $P^{32}$ in milk are illustrated by Fig. 2 The histogram gives the results of the direct measurements in each period.

The average daily secretion of $P^{32}$ in milk as percentage of the injected dose for the first to the 7th day after injection amounted to 7.4, 4.9, 2.8, 2.1, 1.6 and 1.4% respectively.

In a week, cow 798 (Trial III) secreted, in 66 liters of milk, 20% of the injected $P^{32}$; cow 890 secreted, in 58 liters of milk, 23% of the injected dose.

*Discussion.* Cows injected intravenously with 15 and 30 millicuries of $P^{32}$ respectively produced "hot" milk almost immediately. The radioactivity of the milk of both cows reached the same peak at about 5 hours after injection when 1 liter of milk contained 1.2% of the injected $P^{32}$. Doubling the injected dose of $P^{32}$ thus changed neither the time at which the maximum activity in milk was reached nor the relative height of the maximum $P^{32}$ concentration in milk as percentage of the injected dose.

At the time of maximum activity in the milk, the inorganic P (trichloracetic acid filtrate) was relatively more active than the organic P. This conclusion is drawn from the observation that even 10 hours after injection, the organic $P^{32}$ in milk amounted to less than 20% of the total $P^{32}$, whereas during the 5th to 7th week of lactation, according to Van Slyke and Bosworth[2] 20% of the milk P is present in organic form, practically all in casein. Aten and Hevesy[3] have reported such a lag in the specific activity of casein P as compared to inorganic P in goat milk. However, 5 hours after injection, the specific activities were already equalized. Goats may have higher turnover rates than cows. Experiments are under way to get more definite information on the partition of $P^{32}$ among the various constituents of the milk. Despite the lag mentioned, cows are good producers of labeled casein. From milk collected for 3 days after injecting 40 millicuries of $P^{32}$ per cow, we have prepared casein with a radioactivity of 2 microcuries per gram.

The peak of $P^{32}$ activity in the milk of our cows was reached at nearly the same time after intravenous injection as the peak of $Sr^{98}$ in milk of two Holstein cows in the earlier trials of Erf and Pecher (l.c.). $Sr^{98}$, however, reached a higher maximum relative concentration, namely 2.2% of the injected dose per liter milk, and then decreased faster than the $P^{32}$ in our trials. For 4½ days after injection of the $Sr^{98}$, the two cows secreted 8% and 11% respectively of the injected isotope in the milk, whereas in 4 days after injection of $P^{32}$ 15.1 and 17.9% respectively were secreted by our 2 cows.

The different results with Sr (representing Ca) and P might be expected if cows had a greater storage capacity for P than for Ca in terms of the corresponding amounts secreted in milk. Such an explanation, however, would be unsatisfactory, in view of the relative concentrations of these elements in milk and body. According to Van Slyke and Bosworth (l.c.), milk contains 0.103% P and 0.143% Ca. Using figures of Hogan and Nierman[4] for steers, one may estimate that the body of cattle contains 0.72% P and 1.36% Ca. If this estimate is applicable to our cows, then the ratio (P in body : P in milk) is only ¾ of the ratio (Ca in body : Ca in milk).

The different behavior of Sr (substituting for Ca) and P in lactating cows may be explained by the assumption that the P stored in the body is on the average more mobile than the Ca stored. A faster storage of P would explain the lower peak in milk, and a faster exchange of the stored P would account for the subsequent relatively higher level of $P^{32}$ in the milk.

*Summary.* Intravenous injection of 15 and 30 millicuries of $P^{32}$ respectively into 2 lactating cows produced a maximum relative activity per liter of milk of 1.2% of the injected dose at about 5 hours after injection. The relative activity per liter milk is plotted against time after injection and regression equations are presented for interpolating the data. A week after injection, the milk still contains slightly over 0.1% of the injected $P^{32}$ per liter.

Casein with a radioactivity of 2 microcuries per gram was prepared out of milk collected during 3 days after injection of 40 millicuries of $P^{32}$.

A histogram shows the relative rate of $P^{32}$ secretion in milk. In a week, the 2 cows secreted in their milk 20 and 23% respectively of the injected $P^{32}$.

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[2] Van Slyke, D. D., and Bosworth, A. W., *J. Biol. Chem.*, 1915, **20**, 141.

[3] Aten, A. H. W., Jr., and Hevesy, G., *Nature*, 1938, **142**, 111.

[4] Hogan, A. G., and Nierman, J. L., *Univ. Mo. Res. Bull.*, 1927, **107**, 45.

## 16718 P

# Androgenic Secretion by Tumors of the Mouse Adrenal Cortex.

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*From the Department of Anatomy, University of Minnesota Medical School.**

Tumors of the human adrenal cortex may be responsible for either androgenic or estrogenic activity, although it is more common for such neoplasms to give evidence of androgenic secretion.[1] Adenomas of the adrenal cortex of mice may be induced in certain stocks by gonadectomy,[2,3] and in one stock, the NH, they occur spontaneously, primarily in females.[4†] In contrast to the human, the mouse neoplasms have been found to secrete primarily estrogen in most instances.[1-3] It was consequently of great interest to the authors to find that in the Bagg albino stock the majority of tumors induced in mice of both sexes secreted primarily androgenic hormone, although it is likely that both estrogenic and androgenic substances are produced simultaneously.

All of 10 males castrated at weaning age possessed tumors of the adrenal cortex 2 years following operation. In 5 of the 10, the seminal vesicles were very large and filled with secretion; in 4 others the seminal vesicles were developed beyond the castrate type. Submaxillary glands[5] and renal corpuscles[6] were histologically male in most instances.

Ten months following ovariectomy 3 of 4 Bagg albino females had cortical tumors with which there was an associated androgenic activity. The vaginal epithelium was only 3-4 cell layers thick and the superficial lining cells were high columnar, the cytoplasm being filled with mucous. This picture is indicative of combined estrogenic and androgenic secretion.[7] Seminal vesicle grafts[8] of weanling Bagg albino males had been made into both axillae of 2 of the females 57 days before autopsy. All 4 grafts were greatly hypertrophied, and filled with secretion. The renal corpuscles and submaxillary glands of these females were of the male type. The fourth female of this group had cortical tumors of histologic structure similar to that found in the other 3 ovariectomized mice. In this case, however, the reproductive tract showed signs of estrogenic stimulation, and the renal corpuscles and submaxillary glands had not become masculinized.

*Summary.* A majority of gonadectomized male and female mice of the Bagg albino stock developed adrenal cortical tumors which secreted androgenic hormone. There is evidence that both androgenic and estrogenic hormone may be produced simultaneously by these tumors.

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* This investigation has been aided by grants from the National Cancer Institute, The Jane Coffin Childs Memorial Fund for Medical Research, and the Cancer Fund of the Graduate School of the University of Minnesota.

[1] Cahill, G. F., In Endocrinology of Neoplastic Diseases, 1947, Oxford University Press, New York.

[2] Gardner, W. U., *Cancer Research*, 1941, **1**, 632.

[3] Wooley, G., Fekete, E., and Little, C. C., *Endocrinology*, 1941, **28**, 341.

[4] Kirschbaum, A., Frantz, M. F., and Williams, W. L., *Cancer Research*, 1946, **6**, 707.

† The authors have also observed spontaneous tumors of the adrenal cortex in old, intact Bagg albino mice of both sexes and in aged females of the CBA strain.

[5] Lacassagne, A., *C. R. Soc. de Biol.*, 1940, **133**, 180.

[6] Pfeiffer, C. A., Emmel, V. M., and Gardner, W. U., *Yale J. Biol. and Med.*, 1940, **12**, 493.

[7] Korenchevsky, V., and Hall, K., *J. Path. and Bact.*, 1937, **46**, 681.

[8] Hill, R. T., *Endocrinology*, 1937, **21**, 633.

16719

# Testosterone Propionate Minimum for Induction of Male Development in Anurans; Comparative Data from Other Vertebrates.*

BEATRICE MINTZ. (Introduced by Emil Witschi.)

*From Whitman Laboratory of Experimental Zoology, The University of Chicago.*

After the discovery[1,2] that administration of male sex hormone to anuran tadpoles can induce testicular development in genetic females, it became of some interest to study the sensitivity of the system in terms of the smallest quantity of androgen which could effect such a change. In previous investigations in which tadpoles were raised from the time of hatching to metamorphosis in water containing graded concentrations of testosterone propionate, male differentiation irrespective of genetic constitution was observed in *Rana sphenocephala, Rana pipiens,* and *Pseudacris triseriata* at 0.01 mg (10 μg) of hormone per liter of aquarium water, a concentration of 1:100,000,000.[3,4] From all indications, however, it appeared that the minimal effective dose might well be considerably below any so far employed. Such has since been found to be the case (preliminary report by Mintz and Witschi[5]).

*Materials and Methods. Rana sylvatica* eggs were collected near New Haven, Conn. in March and April and treatments were initiated 6-8 days after hatching. Controls preserved at this time were entirely undifferentiated as to sex. Groups of 50-100 larvae, including untreated controls, were each kept in 10 liters of aerated water. Solutions of crystalline testosterone propionate† in 95% ethyl alcohol were added daily from calibrated syringes to the following concentrations in different groups: 1,000, 200, 40, 10, 2, 1, 0.1, and 0.01 μg/liter. The alcohol volume measured 0.15 ml/liter of water. These animals were preserved in Bouin's fixative approximately 30 days later during metamorphosis when one foreleg perforated the opercular covering. Individuals that died were preserved only if they appeared to be still in good condition. The temperature of the amphibian room averaged 20°C but occasionally dropped down to 16°C.

*Results.* As seen in Table I, tadpoles exposed to 2 μg or more of hormone/liter emerge exclusively as phenotypic males indistinguishable from control males, with the exception of 2 male-like intergrades and one female at 10 μg/liter. It is possible that the latter non-conformist was accidentally transferred from one of the lower dosage jars. The amount of 2 μg/liter, still inducing complete masculinization of all genetic females, corresponds to a concentration of 1:500,000,000. One half of this quantity, 1 μg/liter or 1:1,000,000,000, represents the threshold value for masculinization inasmuch as it produces a preponderance of males and masculine intersexes.

Among the controls, we observe a moderate excess of females. In a population of this size, the probability of obtaining a deviation from the expected 1:1 ratio of males : females (actually 58 males : 58 females) due to the error inherent in random sampling would bring the acceptable range to the extremes of

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* The experimental part of this work was conducted at the State University of Iowa and supported by grants from the National Research Council, Committee for Research in Problems of Sex, under the administration of Dr. Emil Witschi. The author wishes to thank Dr. L. V. Domm for providing accommodations for completing the histological portions of the study at Whitman Laboratory.

[1] Gallien, L., *C. R. Acad. Sci.*, 1937, **205**, 375.

[2] Witschi, E., and Crown, E. N., *Anat. Rec. Suppl.*, 1937, **70**, 121.

[3] Witschi, E., *Cold Spring Harb. Sympos. on Quant. Biol.*, 1942, **10**, 145.

[4] Witschi, E., Foote, C., and Mintz, B., *Anat. Rec. Suppl.*, 1942, **84**, 455.

[5] Mintz, B., and Witschi, E., *Anat. Rec. Suppl.*, 1946, **96**, 30.

† The author wishes to thank the Ciba Pharmaceutical Products, Inc., for supplying testosterone propionate (Perandren).

TABLE I.
Sex distribution in *Rana sylvatica.*

| Treatment | ♀ | ♀̇ | ♂ | ♂̣ | ♂ |
|---|---|---|---|---|---|
| Testosterone propionate | | | | | |
| 1000 μg/l | — | — | — | — | 30 |
| 200 ” | — | — | — | — | 38 |
| 40 ” | — | — | — | — | 46 |
| 10 ” | 1 | — | — | 2 | 131 |
| 2 ” | — | — | — | — | 77 |
| 1 ” | 13 | 2 | 3 | 7 | 66 |
| 0.1 ” | 45 | 2 | 4 | 6 | 40 |
| 0.01 ” | 47 | — | 2 | 2 | 37 |
| Controls | 68 | — | 4 | — | 44 |

47 : 69 in favor of either sex, as calculated by 3 P.E. or 2 S.E. The observed distribution of 48 males and intersexes : 68 females falls within these limits but is close to the extremes and, together with the occurrence of 4 hermaphrodites, may therefore indicate a slight deviation toward the semi-differentiated racial type.

*Discussion.* It is logical to assume that the tadpole does not utilize all of the hormone available in its ambient environment. This would certainly appear to be indicated judging from Foote's[6] measurements of the slow rate of disappearance of estrone from the aquarium water in which *Rana pipiens* tadpoles were kept. Although it may well be that the animals are able to concentrate the absorbed hormone to critical levels, we observe at any rate that they exhibit a remarkable ability to "detect" exceedingly minute amounts of androgen to which they respond with a marked alteration in morphogenesis. This testifies to the extreme lability of the embryonic sex inductor system in anurans.

The method outlined might serve as an assay procedure for ascertaining the presence of very small quantities of testosterone not otherwise determinable. A series of experimental groups treated with successively diluted aliquots of the preparation in question would indicate that proportion of the total which contained the minimal effective dose for male differentiation.

Table II summarizes data on sensitivity of some of the more widely studied vertebrates to testosterone propionate. Because of the different modes of hormone administration, the differing extents of changes produced as well as total weight of the test animal, and, presumably, differences in economy in utilizing the hormone, we must of course regard such comparisons with some degree of reservation. The tests cited indicate more recently reported lowest levels at which an effect can still be obtained; credit is not here necessarily given to the originator of the test. Literature on quantitative bioassays has by now become impressive in volume, and includes some disagreement on threshold dosages. For purposes of comparison, the threshold dose for masculinization of *Rana sylvatica* is expressed as μg/ml since this amount of solvent is considerably closer to the volume administered to other test animals than is the "per liter" designation.

In examining these data, it is apparent that the anuran larva displays a much greater reactivity to testosterone propionate as compared with other vertebrates studied, and that the nature of the response, involving the primary sex characters, is a relatively more drastic one.

There are indications of the existence of species differences in response among anurans. Treatment of a small number of Hyla arenicolor larvae with the 2 μg/liter dose resulted in approximately equal numbers of males and females, plus a few hermaphrodites.

*Summary.* An amount of testosterone propionate as small as 1 μg/liter of aquarium water, a cancentration of 1 : 1,000,000,000, is sufficient to induce male development in a

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[6] Foote, C. L., *J. Exp. Zool.*, 1941, **86**, 291.

TABLE II.
Testosterone Propionate, Minimal* Effective Dosages.

| Test animal | Diagnostic effect | Method of administration | No. of days | Dosage | Reference |
|---|---|---|---|---|---|
| Brown Leghorn capon | Comb growth | Inj. (intramus.) daily | 4-5 | 5-25 μg/d | Koch[7] |
| Brown Leghorn capon | ,, ,, | Local application, daily | 10 | .01-.1 μg/d | ,, |
| English sparrow capon | Bill blackening | Inj. (intramus.) daily | 6 | .2 μg/d | Witschi[8] |
| English sparrow capon | ,, ,, | Local application, daily | 4-16 | .06 μg/d† | Pfeiffer, Hooker, and Kirschbaum[9] |
| Rat ♀§ (8 days old) | Prostate | Inj. daily | 6 | 1 μg/d | Price[10] |
| Rat ♂§ (8 days old) | Prostate, seminal vesicles | ,, ,, | 6 | 1 μg/d | ,, |
| Rat ♀, pregnant | Masculinization of secondary sex char's. of ♀ embryos | Inj. once | — | 2500 μg | Greene and Ivy[11] |
| Hamster ♀, pregnant | Masculinization of secondary sex char's. of ♀ embryos | ,, ,, | — | 1000 μg | Bruner and Witschi[12] |
| Mouse ♀, pregnant | Masculinization of secondary sex char's. of ♀ embryos | ,, ,, | — | 1000 μg | Turner[13] |
| *Rana sylvatica* | Male development | Immersed | 30‡ | 0.001 μg/ml | |

* Not all workers on mammals listed have tried doses below those given, so that the minima in some cases may be lower.

† Minimum for testosterone rather than testosterone propionate. Positive results with 2 μg/d of testosterone propionate for 4-5 days but minimum not determined.

‡ The critical time has not been determined. Possibly the results could be read sooner.

§ Hermaphrodite.

[7] Koch, F. C., 1946, personal communication.

[8] Witschi, E., *Anat. Rec. Suppl.*, 1943, **87**, 478.

[9] Pfeiffer, C. A., Hooker, C. W., and Kirschbaum, A., *Endocr.*, 1944, **34**, 389.

[10] Price, D., *Physiol. Zool.*, 1944, **17**, 377.

[11] Greene, R. R., and Ivy, A. C., *Science*, 1937, **86**, 200.

[12] Bruner, J. A., and Witschi, E., *Am. J. Anat.*, 1946, **79**, 293.

[13] Turner, C. D., *J. Morph.*, 1939, **65**, 353.

large majority of *Rana sylvatica* larvae raised in such a solution from hatching to metamorphosis. The relationship of these results to similar work on other vertebrates is discussed.

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The author wishes to acknowledge the assistance of Miss Jane Elchlepp of the Department of Zoology, The State University of Iowa, in the maintenance and treatment of some of the experimental groups.

### 16720

## Concerning the Relation Between Pituitary Adrenocorticotrophin and the Circulating Blood Platelets.

Monte A. Greer and Bruce R. Brown. (Introduced by R. O. Greep.)

*From the Joseph H. Pratt Diagnostic Hospital and the Department of Medicine, Tufts Medical School, Boston, Mass.*

Although it has been known for some time that infection, trauma, hemorrhage, asphyxia, etc. result in rather marked alterations in the number of circulating blood platelets, the exact mechanism controlling the number of platelets is as yet unknown. With the discovery by Dougherty and White[1] that pituitary adrenocorticotrophin caused a dissolution of lymphoid tissue and a peripheral lymphopenia a new line of investigation was opened up. Since the conditions under which changes in the platelets have been reported are those which would stimulate a release of pituitary ACTH, and since this hormone has been shown to affect other blood elements through the adrenal, it did not seem too unreasonable to believe that the platelets might also be at least to some extent under the control of corticotrophin.

Consequently experiments were undertaken in both rats and humans to determine whether any effect of ACTH upon the blood platelets could be demonstrated. As far as could be determined by these experiments, there is no effect upon the platelets of either species by this hormone.

*Experimental.* Sixteen 100-150 g, hooded, male rats were injected subcutaneously with 80 mg of a suspension of acetone-dried hog pituitary after two control counts had been made, and determinations of the platelets* and RBC were made from the capillary tail blood every hour for 8 hours. No significant change was seen to occur in either blood element.

Six rats were injected in a similar manner with 40 mg of hog pituitary powder every morning for 3 days. Again there was no significant change in the number of platelets.

Four rats were injected with 10 mg of a purified pituitary corticotrophin preparation and counts made every hour for 8 hours with similarly negative results.

Following these experiments a preparation of pituitary ACTH† which was quite pure was made available to us, and it was decided

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[1] Dougherty, T. F., and White, A., *Endocrinology*, 1944, **35**, 1.

* Three methods were used to count the platelets: (1) that of Rees and Ecker[2]; (2) a direct chamber count using 3.8% sodium citrate as the diluting fluid; (3) an indirect count of a wet preparation containing platelets stained with brilliant cresyl blue. The direct counts were used chiefly on the rats and the indirect on the human subjects.

[2] Rees, H. M., and Ecker, E. E., *J.A.M.A.*, 1923, **80**, 621.

† Kindly supplied by Dr. John R. Mote of the Armour Laboratories, Chicago, Ill. It was believed that each vial in the lot supplied (Lot G-59703-H) contained the equivalent of 12 mg of the Armour ACTH standard. After the completion of these experiments Dr. Mote informed us that on re-assay each vial contained only the equivalent of 5.2 mg of their standard. The doses given in this paper conform to the final assay.

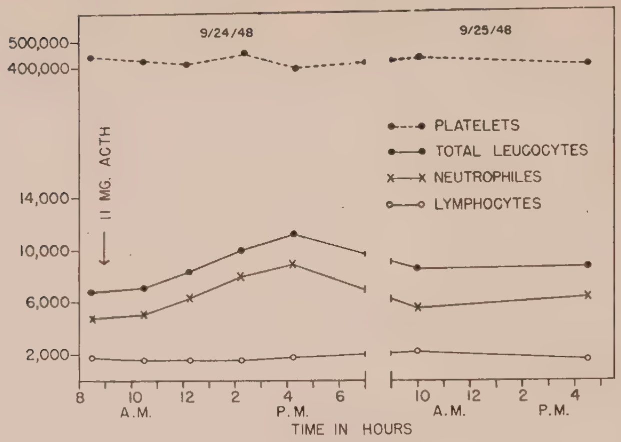

FIG. 1.

The effect of a single, intramuscular injection of 11 mg of ACTH

to test this in human subjects. Following a control determination of all blood elements, 11 mg of this preparation were injected intramuscularly into a normal male subject every 4 hours for 24 hours. A white count, differential, and platelet counts were performed every 2 hours for the first day and in the morning and afternoon of the second day.

The same procedure was followed in a young woman with idiopathic thrombocytopenic purpura who had not had a favorable response to splenectomy.‡ (It was reasoned that an increase in the platelets might show up much more readily in a patient in whom they were quite low originally.)

Two normal young women were given 11 mg of ACTH intramuscularly and the above counting procedure followed.

One young woman with hypopituitarism, which had presumably resulted from postpartum pituitary necrosis 3 years previously, was given 11 mg of ACTH in the morning and 7 mg in the afternoon and counts made as above.

In no instance could any significant change in the number of circulating platelets be observed, although there was a definite and usually quite striking rise in the total white count and the polymorphonuclear leukocytes.

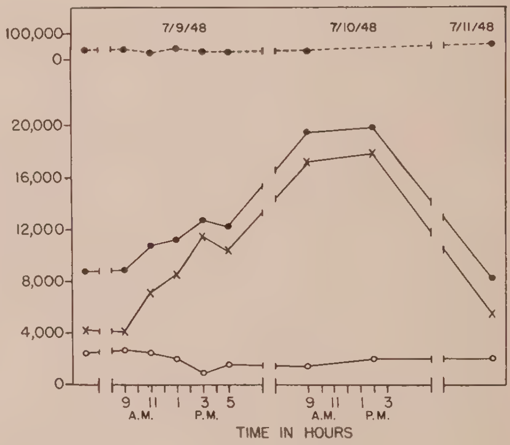

FIG. 2.

The effect of 66 mg of ACTH on a patient with idiopathic thrombocytopenic purpura. 11 mg were given intramuscularly every 4 hours for 24 hours beginning at 8 A.M. July 9. The legend corresponds to Fig. 1.

It is interesting that the number of circulating lymphocytes remained relatively constant, no true lymphopenia being observed in any instance. In the patient with hypopituitarism the increase in the polymorphonuclear leukocytes was much less marked than in the other subjects.

*Summary.* Under the above experimental conditions, no effect of pituitary adrenocorticotrophin upon the number of circulating blood platelets could be detected.

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‡ Patient of Dr. William Dameshek.

### 16721

# Effect of Diisopropyl Fluorophosphate (DFP) on the $QO_2$ of the Rabbit Cerebral Cortex.

SYDNEY ELLIS.* (Introduced by G. S. Eadie.)

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Several reports indicate that diisopropyl fluorophosphate (DFP), or related compounds, may be of clinical value in view of their prolonged inhibition of cholinesterase activity. When injected into humans, stimulation of cholinergically innervated tissues has been noted. The central nervous system also appears to be affected.[1,2] Since 'central' effects have not been noted when neostigmine is administered by the usual routes,[3] or in large doses by continuous infusion,[4] the suggestion has been made that this difference in the action of the two agents is due to the greater fat solubility of DFP in contrast to neostigmine.[1,2]

Another possibility exists, *i.e.*, that DFP, in addition to inhibiting cholinesterase, might affect the metabolism of the brain. This has been investigated in rabbits injected with DFP in doses sufficient to inactivate cholinesterase.

*Methods.* Normal rabbits were sacrificed as controls by injecting air intravenously. Diisopropyl fluorophosphate at a concentration of 1:1000 freshly dissolved in physiological saline was injected intravenously in amounts stated in the table. The animals were used immediately following death or, if not dead 30 minutes after the injection of DFP, they were sacrificed by injecting air intravenously. Immediately after death the cranium was opened and the cerebrum removed intact.

Cerebral cortex slices were removed from one cerebral hemisphere by the tissue slice technic of Deutsch.[5] The remaining hemisphere was homogenized in 0.025 M sodium bicarbonate solution[6] with the Potter all-glass homogenizer and made up to a final dilution of 1:10 with the bicarbonate solution.

Cholinesterase activity was determined by Ammon's method[7] with 0.5 cc of the 1:10 brain homogenate in a final concentration of 0.015M acetylcholine bromide in 0.025M sodium bicarbonate at 38°C. The gas phase was 5% $CO_2$ and 95% $N_2$.

The oxygen uptake, expressed as cu mm oxygen utilized per mg of dry weight of tissue per hour ($Q_{O_2}$), was determined on cortical slices suspended in 3 cc of a salt mixture of the following composition[8]: 124.6 mM sodium chloride; 4.01 mM potassium chloride; 1.04 mM calcium chloride; 0.52 mM magnesium chloride; 10 mM phosphate buffer, pH 7.4; 0.20 g% anhydrous glucose. The dry weights of the tissues were obtained after the tissues were heated at 105°C for at least 16 hours.

The data of Table I demonstrate that DFP does not change the oxygen uptake of cerebral cortex slices of rabbits treated with this compound, as compared with untreated rabbits, even when acetylcholine hydrolysis by the cerebrum is completely inhibited. This does not eliminate the possibility of a more subtle

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* Formerly Captain, Sanitary Corps, A.U.S. Present address: Department of Physiology and Pharmacology, Duke University School of Medicine, Durham, N. C.

[1] Comroe, J. H., Jr., Todd, J., Gammon, G. D., Leopold, I. H., Koelle, G. B., Bodansky, O., and Gilman, A., *Am. J. Med. Sci.*, 1946, **212**, 641.

[2] Grob, D., Harvey, A. M., Langworthy, O. R., and Lilienthal, J. L., Jr., *Bull. Johns Hopkins Hosp.*, 1947, **81**, 257.

[3] Harvey, A. M., Lilienthal, J. L., Jr., Grob, D., Jones, B. F., and Talbot, S. A., *ibid.*, 1947, **81**, 267.

[4] Acheson, G. H., and Ellis, S., unpublished data.

[5] Deutsch, W., *J. Physiol.*, 1936, **87**, 56.

[6] Ellis, S., and Root, M. A., *Fed. Proc.*, 1944, **3**, 70.

[7] Ammon, R., *Arch. ges. Physiol.*, 1930, **233**, 486.

[8] Jandorf, B. J., and Williams, R. H., *Am. J. Physiol.*, 1944, **141**, 91.

TABLE I.
Oxygen Uptake and Cholinesterase Activity of Brain of Normal and DFP-Treated Rabbits.

| DFP mg/kg i.v. | Cholinesterase activity cu mm CO/30 min/ 0.5 cc$^2$ homogenate | $Q_{O_2}$ |
|---|---|---|
| 0.0 | 233 | 11.2 |
| 0.0 | 299 | 10.9 |
| 0.0 | 246 | 9.6 |
| 0.0 | 222 | 10.2 |
| 0.0 | 244 | 9.9 |
| 0.0 | 233 | 10.3 |
| 0.0 | 239 | 10.5 |
| 0.0 | 221 | 9.9 |
| Avg | 242 | 10.3 |
| 0.5 | 4 | 9.9 |
| 0.5 | 4 | 10.2 |
| 0.5 | 4 | 10.6 |
| 1.0 | 4 | 8.8 |
| 1.0 | —0.7 | 10.4 |
| 1.0 | —1.1 | 11.4 |
| Avg | 2 | 10.2 |

All values are averages of duplicate or triplicate determinations.

effect on brain metabolism. It does show, however, that DFP has no appreciable effect on the normal oxygen uptake of cerebral cortical slices, and that cholinesterase activity is not essential for such oxygen uptake.

*Discussion.* Some related studies of the effect of DFP on enzymes other than esterases have been reported. Webb[9] found that the following enzyme systems were not affected by DFP at concentrations of M/500 to M/2000: cytochrome oxidase, succinic dehydrogenase, hexokinase, Robison ester glycolysis by muscle powder, choline, dehydrogenase, catalase, carboxylase, ptyalin, peroxidase, lactic dehydrogenase of muscle, diaphorase, liver alcohol dehydrogenase, and carbonic anhydrase. Hunt[10] has stated that the normal oxygen uptake by liver homogenate and the additional oxygen uptake due to added ethyl butyrate are not inhibited noticeably by DFP.

The increased respiration of rat brain slices observed by Levy[11] when the tissue was in concentrations of eserine salicylate between 1:500 and 1:1000 are attributable to the enormous concentration of the drug. It may be that the salicylate ion is being metabolised at a sufficient rate at this high concentration to produce the increased oxygen uptake attributed to eserine.

*Summary.* When injected into rabbits in lethal doses, diisopropyl fluorophosphate had no appreciable effect on the oxygen uptake of cerebral cortex slices of these animals even though cholinesterase was completely inhibited.

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[9] Webb, E. C., *Biochem. J.*, 1948, **42**, 96.

[10] Hunt, C. C., personal communication.

[11] Levy, J., *Bull. soc. chem. biol.*, 1946, **28**, 338.

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### 16722 P

## *In vitro* Studies of a Growth Inhibitory Fraction Obtained from Adult Sheep Cardiac Muscle.*

R. S. HOFFMAN, JAMES A. DINGWALL AND WILLIAM DEW. ANDRUS.
(with the technical assistance of Irene Jacob.)

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In earlier papers[1,2] we have reported on the growth stimulating effects of a Tyrode's solution extract of animal tissue as demonstrated both *in vitro* and when applied topically to wounds in dog and man. In the course of attempts to fractionate adult animal tissue extracts in order to determine the factors responsible for this stimulation, one crude fraction has been of particular interest because it appeared to have inhibitory effects.

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* This work was supported by a grant furnished by the Committee on Growth of the National Research Council.

[1] Hoffman, R. S., and Dingwall, J. A., *Surg., Gynec. and Obst.*, 1944, **79**, 103.

[2] Hoffman, R. S., Dingwall, J. A., and Andrus, W. DeW., *Ann. Surg.*, 1946, **124**, 1125.

This fraction was prepared by extracting finely minced sheep cardiac muscle with acetone in the cold, evaporating the solvent, lyophilizing and dissolving the dry residue in 10 volumes of Tyrode's solution. From 660 g of fresh cardiac muscle the yield was approximately 10 g of residue. The following is a report on preliminary experiments dealing with the effects of this material.[†]

*Effect on Normal Cells.* Third passage hanging-drop cultures of chick heart fibroblasts were divided and one half of each culture was used as the test object, the other half as the control. Each was planted in a separate D-3.5 Carrel flask containing 0.5 cc of chicken plasma, 1 cc Tyrode's solution and one drop of chick embryonic extract. After 48 hours 0.5 cc of the test solution was added as a supernatant layer to the test culture and 0.5 cc of Tyrode's solution to the control. The pH of both control and test cultures was adjusted to from 6.5 to 7.0. The effect of the test solutions as compared to the controls was observed for from 2 to 4 days.

The residue of the acetone extract of the sheep heart, redissolved in Tyrode's solution, markedly inhibited the growth of chick fibroblasts as no increase whatsoever in surface area was observed after this material had been added in dilutions up to 1:500. The cells, however, were not killed, for when the cultures were washed with Tyrode's solution and transplanted into fresh medium they began to grow and to maintain a rate of growth eventually equaling that of the controls.

*Effect on Tumor Cells.* To test the effects of this extract on tumor cells, cultures of a mouse breast tumor were also studied. This neoplasm arose spontaneously in a C3H mouse 2 years ago, and while originally exhibiting all the features of a mammary adenocarcinoma, it has gradually become sarcomatous in character.[‡] The tumor was cultivated in Carrel flasks in the same type of coagulum used for the normal cells, but 0.3 cc of rabbit serum and 0.4 cc of mouse embryo extract were added as a supernatant fluid phase. The latter was changed every 4 days and the cultures transplanted to other Carrel flasks every 10 days.

Second passage cultures were divided into equal parts and the halves were planted in a separate Carrel flask to be used either as test or control objects. All cultures were maintained in medium 4 or 5 days, during which time satisfactory growth of the tissue was observed. On the fourth or fifth day, the supernatant fluids were removed from all the cultures and replaced in the experimental series with the residue from the acetone solution that had been redissolved in Tyrode's solution to give a final dilution of 1:500. In the sister halves, or controls, 0.5 cc of Tyrode's solution was added. A complete cessation of growth was obtained in the tumor cultures treated with extract, whereas the sister cultures continued to show good growth.

*Effect on Bacteria.* The effectiveness of certain animal tissues in producing bacteriostasis and in converting *Staphylococcus aureus* R into an avirulent form has been reported by Nutini and coworkers in several publications;[3-6] and in view of these findings it is of interest to examine the effect of our acetone extract on certain bacteria. One gram of the crude residue was dissolved in 10 cc of Tyrode's solution and applied in serial dilution to standard cultures of organisms. It inhibited the growth of tubercle bacilli in the Tween albumin medium of Dubos and Davis[7] in further dilution of 1/40 and was effective against the same organism cultivated in oleic acid medium when the 10% solution of the crude material was further diluted 1/160.

Complete inhibition of growth of H Streptococcus C 203 occurred when the 10% Tyrode's solution was diluted 1/16, Staphylococcus was inhibited in dilution of 1/32 (crude material 1/320) and *E. coli* at 1/16.

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[†] The crude material was prepared by The Armour Laboratories.

[‡] H. Toolan, personal communication.

[3] Nutini, L. G., and Kreke, C. W., *J. Bact.*, 1942, **44**, 661.

[4] Nutini, L. G., Kreke, C. W., and Scroeder, Gr. M. P., *J. Bact.*, 1943, **50**, 177.

[5] Nutini, L. G., and Lynch, E. M., *J. Exp. Med.*, 1946, **84**, 247.

[6] Nutini, L. G., and Lynch, E. M., *J. Bact.*, 1946, **52**, 681.

[7] Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, **83**, 409.

Other investigators[8–11] have reported the extraction of substances inhibiting cell growth from liver and other sources by methods different from those reported here, and it is not yet clear whether the substance which we have used corresponds in its physical and chemical properties to any of those previously described.

*Summary.* Acetone extract of sheep cardiac muscle lyophilized and redissolved in Tyrode's solution inhibits the growth of chicken fibroblasts and mouse breast tumor cells *in vitro*. A similar inhibitory activity of varying degree is exhibited against standard strains of tubercle bacilli, hemolytic *Streptococcus aureus* and *E. coli*.

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[8] Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1925, **42**, 143.

[9] Heaton, T. B., *J. Path. and Bact.*, 1926, **29**, 293; 1929, **32**, 565.

[10] Shebruja, T., Inaba, M., and Kawaguchi, A., *Tr. Japan. Path. Soc.*, 1935, **25**, 412.

[11] Brues, A. M., Subbarow, Y., Jackson, E. B., and Aub, J. C., *J. Exp. Med.*, 1940, **71**, 423.

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## 16723

# Effect of Adrenal Cortex Extract Upon the Urinary Nitrogen of Rats Following Adrenalectomy.

DWIGHT J. INGLE AND MILDRED C. PRESTRUD.

*From the Research Laboratories, The Upjohn Company, Kalamazoo, Mich.*

The adrenal cortex has been characterized as a regulator of protein metabolism. It is generally true that the adrenally insufficient animal excretes smaller than normal amounts of urinary nitrogen and that the administration of adrenal cortex extract or of the 11-oxy steroids favors an increased loss of nitrogen[1]. The present report concerns an exception to the above rule. Others are known.[2–5]

In studies on the force-fed rat it was noted[4,5] that following adrenalectomy the animals which were given saline to drink without additional treatment excreted more urinary nitrogen than did similar animals which were treated with adrenal cortex extract or than did sham-operated rats. The present study confirms and extends these preliminary observations.

*Methods.* Male rats of the Sprague-Dawley strain were maintained on stock diets of Purina Dog Chow (Experiment 1) and Archer Dog Pellets (Experiment 2). When the rats reached a weight of approximately 300 g they were adapted to the force-feeding of a medium carbohydrate diet by stomach tube each morning (8:30 to 9:15 A.M.) and afternoon (4:15 to 5:00 P.M.). The technic of force-feeding and the diet (Table I) used

TABLE I.
Medium Carbohydrate Diet.

| Constituent | G |
|---|---|
| Cellu flour (Chicago Dietetic Supply) | 120 |
| Osborne & Mendel salt mixture | 40 |
| Dried yeast (Pabst) | 100 |
| Wheat germ oil | 10 |
| Cod liver oil | 10 |
| Vitamin K (2-methyl-1,4-naphthoquinone) | 100 mg |
| Mazola oil | 200 |
| Casein (Labco) | 160 |
| Starch | 200 |
| Dextrin | 190 |
| Sucrose | 200 |
| Water to make total of | 2000 cc |

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[1] Long, C. N. H., Katzin, B., and Fry, E. G., *Endocrinology*, 1940, **26**, 309.

[2] Koelsche, G. A., and Kendall, E. C., *Am. J. Physiol.*, 1935, **113**, 335.

[3] Berman, D., Sylvester, M., Hay, E. C., and Selye, H., *Endocrinology*, 1947, **41**, 258.

[4] Ingle, D. J., and Oberle, E. A., *Am. J. Physiol.*, 1946, **147**, 222.

[5] Ingle, D. J., Ward, E. O., and Kuizenga, M. H., *Am. J. Physiol.*, 1947, **149**, 510.

were modifications of those described by Reinecke, Ball, and Samuels.[6] The rats were brought to a full feeding of 26 cc per rat per day on the 5th day. After the rats had been force-fed for 2 weeks the adrenal glands were removed by the procedure of Ingle and Griffith.[7] In the control animals the adrenal glands were exposed but were not damaged. Asepsis was successfully maintained in these operations. All of the animals were given a solution of 1% sodium chloride to drink during all phases of the experiments. The animals were housed in an air-conditioned room in which the temperature was maintained at 74 to 78°F and the humidity at 30 to 35% of saturation.

Twenty-four-hour samples of urine were collected at the same hour each day and were preserved with thymol with added citric acid (1 g per sample) to insure the acidity of the urines for nitrogen analysis. The determination of urinary nonprotein nitrogen was by the micro-Kjeldahl procedure as follows: proteins were precipitated as the salts of tungstic acid by the Folin-Wu procedure. The organic matter was oxidized by sulfuric acid and hydrogen peroxide. Copper sulphate and sodium sulphate were used as catalysts. The ammonia was distilled off into a standard acid solution and titrated with standard base.

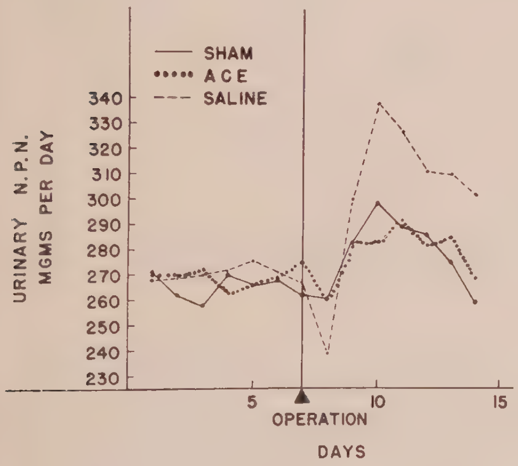

Fig. 1.
The effect of adrenal cortex extract upon the level of urinary nitrogen immediately following adrenalectomy. Averages of 8 rats per group.

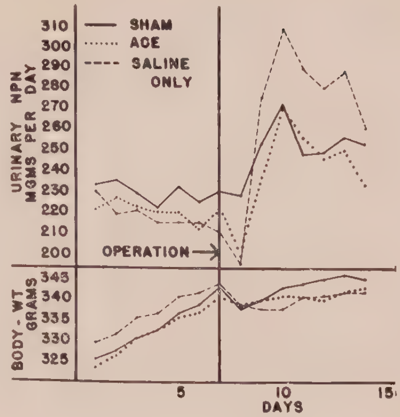

Fig. 2.
The effect of adrenal cortex extract upon the level of urinary nitrogen immediately following adrenalectomy. Averages of 10 rats per group.

*Experiments and results.* In Experiment 1 (Fig. 1) 24 rats were observed during a control period of 2 weeks. At this time 8 rats were adrenalectomized and were given 4 cc of beef adrenal extract (Upjohn) per day for 7 days; 8 adrenalectomized rats received injections of 4 cc of 0.9% sodium chloride solution for 7 days; and 8 sham-operated rats received injections of 4 cc of 0.9% sodium chloride solution for 7 days. The adrenalectomized rats showed some decrease in urinary nitrogen during the first 24 hours with a subsequent rise. The increase in nitrogen loss was significantly greater in the saline-injected adrenalectomized animals than in either the treated adrenalectomized group or the sham-operated controls.

Experiment 2 (Fig. 2) was identical with Experiment 1 except that 30 rats were used and it was carried out during the summer months instead of early winter. The relative changes following operation were identical with the results of Experiment 1. The level of urinary nitrogen was significantly lower than for Experiment 1 during all phases of the experiments.

*Discussion.* These data support our earlier observations[4,5] that the administration of adrenal cortex extract to force-fed rats immediately following adrenalectomy tends to suppress the post-operative increase in urinary nitrogen as compared with that of the untreated animal. The adrenalectomized rat usually shows a temporary decrease in urinary nitrogen which is not shown by sham-operated animals. This may possibly reflect a difference in the breakdown of thymus and other lymphoid tissue or it may reflect an increase in nitrogen retention in the body fluids. Noble and Toby[8] reported a temporary decrease in loss of nitrogen after adrenalectomy followed by a rise above the pre-operative values.

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[6] Reinecke, R. M., Ball, H. A., and Samuels, L. T., Proc. Soc. Exp. Biol. and Med., 1939, **41**, 44.

[7] Ingle, D. J., and Griffith, J. Q., Chapter 16, The Rat in Laboratory Investigation, J. B. Lippincott Co., Philadelphia, 1942.

Koelsche and Kendall[2] found that adrenal cortex extract tended to inhibit the catabolic effect of thyroxin in the adrenally insufficient dog. Berman *et al.*[3] noted that regeneration of liver is suppressed in the adrenalectomized rat and that regeneration is supported by treatment with adrenal cortex extract.

The possibility must be considered that adrenal cortex extracts favor anabolism under certain conditions because of the presence of androgenic steroids. However, such extracts have never been shown to possess androgenic properties.

The average level of urinary nitrogen was higher in all phases of Experiment 1 than in Experiment. 2 Such shifts in the average level of constituents of blood, urine and tissues are not uncommon in this laboratory. The reasons have never been elucidated beyond the demonstration that even small variations in temperature are important. Values on the nitrogen content of our fluid diets, on standards and unknowns have remained uniform. Because unknown variables influence the results of experiments which are thought to be under rigid control, it is essential that all control studies be done in parallel with the experimental study.

*Summary.* Two experiments were carried out as follows. Force-fed rats given saline to drink were subjected to adrenalectomy or to sham operations. Half of the adrenalectomized rats were treated with 4 cc of beef adrenal extract per day. All of the animals showed an increase in urinary nitrogen following operation but it was significantly less in those animals which either had their adrenal glands intact or were treated with adrenal cortex extract.

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[8] Noble, R. L., and Toby, C. G., *J. Endocrinol.*, 1948, **5**, 303.

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### 16724

## Augmentation of Femoral Venous Flow in the Dog by Electrical Stimulation of Muscles.

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*From the Department of Surgery, Western Reserve University, Cleveland, Ohio.*

The procedure was performed 6 times on 2 mongrel dogs, weighing approximately 11 kg. They were anesthetized with intravenous nembutal. Eighteen mg of heparin was given intravenously at the beginning of the procedure. The femoral vein was exposed and cannulated. On one dog a straight glass cannula was used. On the other a T-shaped cannula was inserted, so that no backing up of blood was allowed between periods of flow. The diameter of the T-cannula was smaller than that of the straight one; consequently the minute flow was less. The blood was caught in a sterile graduated cylinder, filtered and reinfused at the end of the experiment.

The stimulation was achieved by means of faradic current. The intensity of the current and the placing of the electrodes on the limb were so arranged that a gentle contraction of the muscles of the whole limb was obtained. The stimulation was intermittent, 2 seconds of exercise being alternated with 3 seconds of rest. The total period of repeated stimulation was 30 seconds and the flow per minute was calculated.

The flow of blood from an open free flowing cannula was caught and measured, both during a period of control when the limb was resting and flaccid, and during a period of stimulation as described above. Near the end of one of the procedures, the cannula became partially occluded by clotted blood, and the flow was reduced to a slow drip. The flow during both exercise and rest was also recorded under these circumstances. Several observations were recorded on each dog, and averages were computed as shown in the tables. Measurements during stimulation were alternated with measurements during rest in an effort to eliminate after-effects of stimulation on blood vessels.

TABLE I.

| No obstruction | At rest cc | With stimulation cc |
|---|---|---|
| Dog No. 1 | 45 | 90 |
| | 39 | 79 |
| | 33 | 74 |
| Avg | 39 | 81 |
| Dog No. 2 | 30 | 53 |
| | 27 | 50 |
| Avg | 28.5 | 51.5 |

*Results.* Measurements are recorded in the table. The venous return was increased from 80 to 100% by stimulation of the muscles when the cannula was flowing freely. When there was a partial obstruction, the increase was considerably greater, in one observation.

*Conclusions.* From these observations it is concluded that the venous flow of blood from a limb is increased by muscular contractions elicited by periodic electrical stimulations. The increase in flow was from 80 to 100%. Results suggest that increased blood flow is one of the reasons why similar methods, when applied to a large number of postoperative patients, have effected a marked reduction in the incidence of venous thrombosis.

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[1] Krogh, August, The Anatomy and Physiology of the Capillaries, New Haven, Yale University Press, 1927.

[2] Bülbring, Edith, and Burn, J. H., *J. Physiol.*, 1939, **95**, 203.

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## 16725

## Protection of Gastric Mucosa of the Rat against Ulceration by Prefeeding with Protein Hydrolysates.

Harry Shay, Margot Gruenstein, Herman Siplet, and S. A. Komarov.

*From Samuel S. Fels Research Institute, Temple University School of Medicine, Philadelphia, Pa.*

The beneficial effects which Co Tui and his associates[1] reported to have resulted from the addition of large amounts of protein hydrolysates to the dietary of patients with gastric or duodenal ulcers have been amply corroborated by subsequent clinical experience. However, no adequate explanation of the action of such preparations has as yet been found. It has been thought that the buffering action[2] of the protein hydrolysates and/or the resultant improvement in the nitrogen balance[1,3,4] may be responsible for the beneficial effects observed. Opinions based on clinical studies have been expressed in favor of each of the above mechanisms, but the evidence presented cannot be regarded as conclusive. While the administration of protein hydrolysate to ulcer patients has given encouraging clinical results, the effect of the hydrolysate on peptic ulcer and the mechanism of its action, as far as we know, have not been investigated experimentally.

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[1] Co Tui, Wright, A. M., Mulholland, J. H., Galvin, T., Barcham, I., and Gerst, G. R., *Gastroenterology*, 1945, **5**, 5.

[2] Levy, J. S., and Siler, K. A., *Am. J. Digest. Dis.*, 1942, **9**, 354.

[3] Hodges, H. H., *Gastroenterology*, 1947, **8**, 476.

[4] Kenamore, B., Lonergan, W., and Shy, J. C., *Gastroenterology*, 1948, **10**, 177.

We have described a method for the uniform production of ulceration in the rumen of the rat.[5] With this method ulceration develops as a result of the action of acid gastric juice accumulating in the empty stomach after pyloric ligation. In addition, a certain minimal concentration of pepsin and of acid is necessary for the development of ulceration, since neutralization of the acid or inactivation of the pepsin prevents ulceration.[5] We realize that rumen ulcers in the rat represent lesions of the wall of a cavity lined with squamous epithelium which in their pathogenesis may differ somewhat from ulcers of the glandular mucosa. However, as we[5] have pointed out previously, this method is valuable in studying the resistance of the gastric mucosa to acid-pepsin ulceration under various experimental conditions. This method has been employed by others[6-9] as well as by ourselves[10] for assaying antacid and anti-ulcer agents. By its means we have studied the effect of protein hydrolysates on the development of experimental ulcers in the rat. In this communication we report results which indicate that the fortifying of an otherwise adequate diet with protein hydrolysate affords definite protection to the gastric mucosa of the rat against the ulcerative action of acid gastric juice.

*Experimental method and results.* In the present study 106 rats were used. All the animals were females of the Wistar strain, bred in our own colony. After weaning, they were maintained on the Rockland Diet.* We confined our investigation to one sex in these experiments on account of the difference in the weight curves of the two sexes, and particularly since Zucker and Zucker[11] have shown that the more rapidly growing male requires more protein in the diet than the female. At the beginning of the experiments the weights of the animals ranged from 100 to 120 g. The females in each litter were divided into 3 groups, one control and 2 experimental. All the animals were fed the Rockland Diet *ad libitum* and the amount of food consumed by each animal during the experimental period of 28 days was recorded.

Two commercial preparations of protein hydrolysate were used as dietary supplements, each in a separate experimental series. Thus, in addition to the colony diet, animals in one series received by stomach tube 2 cc of hydrolysate A† in the morning and also in the late afternoon 6 days a week for a period of 4 weeks (28 days). The animals in the second experimental series received similar 2 cc quantities of a suspension of hydrolysate B,‡ which has a nitrogen content equivalent to that of hydrolysate A. Since hydrolysate A contains 22% of sucrose, a similar solution of sucrose alone was administered as a supplement to the animals in the control series to substitute for the protein hydrolysate. The experiments were done on 10 animals at a time, namely, on 5 which had received supplemental hydrolyzed protein and on 5 controls. All the animals treated similarly have been grouped together in one series and the different series will be referred to as hydrolysate A-treated, hydrolysate B-treated, and sucrose-treated controls respectively.

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[5] Shay, H., Komarov, S. A., Fels, S. S., Meranze, D., Gruenstein, M., and Siplet, H., *Gastroenterology*, 1945, **5**, 43.

[6] Wick, A. N., Irish, A. J., Pauls, F., and MacKay, E. M., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 40.

[7] Pauls, F., Wick, A. N., and MacKay, E. M., *Gastroenterology*, 1947, **8**, 774.

[8] Risley, E. A., Raymond, W. B., and Barnes, R. H., *Am. J. Physiol.*, 1947, **150**, 754.

[9] Morris, C. R., Grossman, M. I., and Ivy, A. C., *Am. J. Physiol.*, 1947, **148**, 382.

[10] Shay, H., Komarov, S. A., Siplet, H., and Gruenstein, M., *Am. J. Digest. Dis.*, 1947, **14**, 99.

* The "Rockland Rat Diet" contains 24.83% of protein, 4.67% of fat, and 49.32% of carbohydrate, according to the statement of the manufacturers (Arcady Farms Milling Company).

[11] Zucker, T. F., and Zucker, L., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 136.

† Hydrolysate A, supplied by Sharp and Dohme, Inc., contains an equivalent of 35% of protein, as calculated from the nitrogen content (N × 6.25), and in addition 22% of sucrose. Control sucrose solution contained added vitamins present in hydrolysate A.

‡ Hydrolysate B, supplied by Mead, Johnson and Co., contains an equivalent of 75% of protein, as calculated from the nitrogen content (N × 6.25). Caloric value: 1 g = 3.75 C.

Some modifications of our previously described technic were adopted in this study. Following the suggestion of Pauls, Wick and Mackay,[7] we used brief ether anesthesia during the operative period, not supplementing it with urethane as we had done in our original studies.[5] This resulted in more rapid recovery of the animal, a much earlier restoration of active gastric secretion, and a more rapid production of ulcers (97% ulceration in 6 hours when 2 cc of saline were instilled into the stomach after pyloric ligation).

The animals in all 3 series underwent the same preliminary treatment, which was as follows. After 48 hours' starvation the animal was placed under light ether anesthesia and the pylorus was ligated. The stomach was lavaged with distilled water, warmed to 38°C, and 2 cc of saline were then introduced. Each animal was placed in a separate clean cage, and was sacrificed 6 hours later.

In this study we routinely inspected the full length of the esophagus in each animal. We believe this to be essential, for in some cases perforating ulcers of the esophagus develop rather early. This causes a large part of the gastric juice to be lost from the stomach, and as a result the residual volume of gastric juice within the stomach becomes so small that it is insufficient to bathe the rumen and cause ulceration. A few cases of perforating esophageal ulcers occurred in our experiments and these were discarded.

Table I shows the number of animals with rumen ulceration and the mean values for the composition of the gastric contents of the animals in each series. It is clear that the fortifying of the already adequate diet with protein hydrolysate produced a rather striking increase in the resistance of the rumen mucosa to the destructive action of the very potent gastric juice. In a considerable pro-

TABLE I.

Incidence of Rumen Ulcers and Composition of the Gastric Contents in Rats Pretreated with Protein Hydrolysates and in Sucrose-Treated Controls.

| Series | Ulcer incidence | | Composition of gastric contents (mean values) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | No. of animals* | % | Animals used† | Volume (ml/hr per 100 g body wt) | pH | Acidity Total (m.eq/l) | Acidity Free (m.eq/l) | Cl (m.eq/l) | Pepsin Conc. (Mett units) | Pepsin Act. (Mett units) |
| Sucrose control | | | | | | | | | | |
| Total | 35 | | 32 | .801 | 1.19 | 80 | 67 | 157 | 81 | 10.7 |
| With ulcers | 34 | 97 | 31 | .803 | 1.19 | 80 | 67 | 157 | 80 | 10.8 |
| Without ulcers | 1 | 3 | 1 | .741 | 1.05 | 94 | 82 | 166 | 108 | 9.6 |
| Amisate treated | | | | | | | | | | |
| Total | 46 | | 44 | .840 | 1.15 | 86 | 71 | 158 | 77 | 10.4 |
| With ulcers | 28 | 59 | 26 | .854 | 1.15 | 85 | 71 | 157 | 80 | 10.3 |
| Without ulcers | 18 | 41 | 18 | .783 | 1.14 | 87 | 71 | 158 | 73 | 10.5 |
| Protolysate treated | | | | | | | | | | |
| Total | 30 | | 30 | .868 | 1.14 | 88 | 75 | 156 | 61 | 7.1 |
| With ulcers | 22 | 73 | 22 | .898 | 1.14 | 86 | 73 | 156 | 65 | 7.3 |
| Without ulcers | 8 | 27 | 8 | .793 | 1.11 | 94 | 82 | 156 | 49 | 6.5 |

* % of animals with ulcers in each series was calculated from all animals used.

† For analysis of gastric contents several animals were not available due to accidents or heavy contamination of the gastric contents with blood.

TABLE II.
Severity of Ulceration in Protein Hydrolysate-Treated* Rats and in Sucrose-Treated Controls.

| Series | No. of animals | Rumen lesions per animal |  |  |  | P‡ |
|---|---|---|---|---|---|---|
|  |  | Single mean | Coalescent mean | Mean | Total S.D.† |  |
| Sucrose-treated controls | 34 | 6.2 | 8.5 | 14.7 | 11.60 | 0.003 |
| Protein hydrolysate-treated* | 50 | 4.2 | 4.6 | 8.8 | 8.30 |  |

* Amisate and protolysate treated series combined.
† Standard deviation.
‡ Probability of both groups belonging to same population.

TABLE III.
Average Daily Food Consumption and Caloric Intake and Average Weight Gain per Animal During Experimental Period (28 Days).

| Series | No. of animals |  | Food intake (g) |  |  | Caloric intake | Wt (g) |  |
|---|---|---|---|---|---|---|---|---|
|  |  |  | Protein | Carbohydrate | Fat |  | Initial | Gain |
| Sucrose-treated (control) | 32 | *a* | 3.27 | 6.51 | 0.61 | 47 | 110 | 38.8 |
|  |  | *b* | — | 0.68 | — |  |  |  |
|  |  | *c* | 100% | 100% | 100% |  |  |  |
| Amisate-treated | 44 | *a* | 2.90 | 5.79 | 0.55 | 47 | 111 | 43.7 |
|  |  | *b* | 1.17 | 0.68 | — |  |  |  |
|  |  | *c* | 125% | 89.9% | 90.1% |  |  |  |
| Protolysate-treated | 30 | *a* | 2.89 | 5.72 | 0.54 | 44 | 111 | 38.9 |
|  |  | *b* | 1.16 | — | — |  |  |  |
|  |  | *c* | 124% | 79.8% | 88.4% |  |  |  |

*a*: Intake with Rockland diet.
*b*: Intake with hydrolysate (values for protein equivalent calculated from nitrogen content (N × 6.25)).
*c*: Total intake expressed as percentage of intake of sucrose-treated control series.

portion of the protein hydrolysate-treated animals, namely, 41% in the hydrolysate A-treated series and 27% in the hydrolysate B-treated series, no ulceration developed, while of the control animals only 3% had no ulcers. This difference was found to be highly significant statistically. Furthermore, those animals in the hydrolysate-treated series that did develop ulcers showed significantly fewer lesions per animal than the animals in the sucrose-treated series (Table II).

The adopted classification of the rumen lesions was, from the very nature of the ulcerative process, somewhat arbitrary. However, under magnification of 10.5 X, the differentiation between "single" and "coalescent" multiple lesions is not difficult. The former are characterized by even, circular margins; the latter by scalloping or irregularity in outline and, as a rule, by greater dimensions. The relatively greater reduction in the "coalescent" ulcers in comparison with single lesions in the hydrolysate treated animals increases the significance of the results.

The most striking feature of these experiments is the absence of any significant differences between the hydrolysate A- or the hydrolysate B-treated series and the sucrose-treated controls in respect of the volume, acidity, and peptic power of the gastric contents. The difference in the incidence of ulceration manifestly cannot be attributed to concurrently lower digestive power of the gastric juice in the hydrolysate-treated animals. We believe that our results indicate this effect of protein hydrolysate to represent a true increase in the mucosal resistance and that it is to be distinguished from the protective effect obtained with other agents, *e.g.*, antacids, which act by depressing the digestive power of the gastric juice. The increased resistance of the mucosa in the hydrolysate-treated animals was not due to a greater caloric intake, since the average amounts of food consumed daily by the animals in all series were isocaloric, although the diets differed in composition. This is illustrated by the data presented in Table III, which also shows the proportions of protein, carbohydrate, and fat in the different diets, so that the food intake of the hydrolysate A- and the hydrolysate B-treated series may be compared with that of the sucrose-treated control series. The rate of growth was adequate in all groups and no statistically significant difference was found between the weight gain of the sucrose-treated controls and that of the protein hydrolysate-treated animals.[§] This indicates that the protein in the Rockland Diet was adequate for the animals at the weight levels at which the experiments were started, and that the addition of protein hydrolysate did not result in any significant increase in the nutritional value of this diet.

Nevertheless, these experiments show that the prefeeding of approximately 25% more hydrolyzed protein to the experimental animals than was fed to the controls for the relatively short period of 28 days resulted in a very marked increase in the resistance of the rumen mucosa to peptic ulceration. This may be regarded as a true prophylactic action. The only other preparation that has been reported to be effective in the prevention of peptic ulceration is enterogastrone, as demonstrated in experimental studies on dogs.[12]

It is noteworthy that this prophylactic effect of protein hydrolysate was exerted against the ulcerating action of acid and pepsin, the agents which were shown to be responsible for the type of experimental ulcer produced by our method in the rat, and which also are generally accepted as factors of primary importance in the development of peptic ulceration in man. All concepts regarding the etiology of gastric and duodenal ulcers in man recognize that factors other than acid and pepsin are also involved. Among these are included differences in the resistance of tissues to the destructive action of gastric juice. If the prophylactic action of protein hydrolysate, which is demonstrated in this study for the the rat, should be found to apply also to man, the first step in the solution of the all-important problem of ulcer prevention will have been accomplished by the simple expedient of fortifying the diet. Experiments are now being carried out to determine whether the addition of native proteins to the diet will produce the same effect as hydrolysates.

*Summary.* The supplementing of an already adequate diet with protein hydrolysates in amounts equivalent to 25% of additional protein produced in rats a marked increase in the resistance of the gastric rumen to peptic ulceration without concurrent changes in the volume, the acidity, or the peptic power of the gastric contents. This protective effect was not accompanied by any significant changes in the rate of growth of these animals as compared with animals on a control diet supplemented with sucrose alone.

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[§] Hydrolysate A-treated *versus* Hydrolysate B-treated: $t = 1.85$, d. f. $= 75$, $P = 0.06$. Hydrolysate A-treated *versus* sucrose-treated control: $t = 1.64$, d. f. $= 81$, $P = 0.10$. Hydrolysate B-treated *versus* sucrose-treated control: $t = 0.02$, d. f. $= 64$, $P = >0.55$.

[12] Hands, A. P., Greengard, H., Preston, F. W., Fauley, G. B., and Ivy, A. C., *Endocrinology*, 1942, **30**, 905.

### 16726

## Technical Procedures for the Study of Organogenesis *in vitro* in Amblystoma.

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The purpose of this report is to present the technical procedures which have been developed in a study of the organogenesis and differentiation of small pieces of embryonic amphibian tissue. Explants from larval *Amblystoma punctatum* (*Ambystoma maculatum* (Shaw) ) were used. The methods employed were modifications of existing tissue culture technics.

Among the many studies of explanted tissues *in vitro,* relatively few have dealt with potentialities and capabilities of explants to differentiate into formed structures, such as organs or organ systems. Fischer[1] first reported endodermal epithelial aggregates which differentiated into cellular structures of the alimentary tract. These structures, called *intestinal organisms,* have been reported occasionally since. Using the Ekman technic of explantation to tap water, Stöhr[2] reported several cases where heart-like structures developed from Bombinator explants. Some of these beat rhythmically within their epithelial sacs. Examination of published photographs of these experiments reveals little in the way of histological differentiation, or of the formation of anatomically proper lumina. All were very small. (Technical details are lacking in the report of Stöhr.) True morphogenesis has been demonstrated by Fell[3] in a series of remarkable papers dealing with the developmental mechanics of bones of the chick *in vitro.* Fell succeeded in effecting the development, in an essentially normal manner, of rudiments of femur, sternum and limb joints from explants of 4 to 9 day avian embryos. Holtfreter[4] has reported, in connection with his extensive studies on early amphibian morphogenesis, that certain pieces of head ectoderm and mesoderm, reared in isolation in salt solution (Holtfreter solution[5]) developed balancers, in some cases more than would normally have formed *in situ.* Discussion of the experimental results of our investigation will be reserved for future publication.

The technical details reported here readily fall into 3 topics and will be taken up in the order of their use in the experiments. The various technics to be discussed may well find application in many fields of investigation.

1. *Sterilization of tissue.* The cultivation of amphibian tissues, particularly those covered with epidermis, has always been hindered by the deleterious influence of the bacterial flora of the pond or tap water in which the animal or embryo is living. Larvae and adults are further contaminated by the passage into their immediate aqueous environment of their intestinal (bacterial) flora. In the past, this has limited the culture of amphibian surface and sub-surface tissues to short term studies or to the fortuitous occurrence of bacteria-free explants. Irradiation of amphibian tissue by ultraviolet light, with the aim of rendering tissues aseptic, always involves the danger of altering the tissue in some manner. The ideal method of rendering tissues sterile requires massive inhibition and death of contaminant bacteria, while the tissue to be studied is left in a normal or close to normal state.

The recent report of Detwiler, Copenhaver and Robinson[6] on the increased survival of amphibian eggs and embryos, following massive operations, when kept in a solution of 0.5 to 1.0% sodium sulfadiazine is of great value to the experimental microsurgeon. We have been able to confirm this finding. However, Detwiler and his associates were concerned with essentially whole animals, which presumably are endowed with a certain amount of tolerance or resistance to bacterial attack. Such a situation does not prevail in the case of excised pieces of organs and tissues of these animals. Not only is the orderly integrative effect of the "whole" animal lost, but also the ratio of wound surface to mass is increased. When such pieces as limb buds are transferred to nutrient medium following treatment in 0.5-1.0% sodium sulfadiazine, the onset of bacterial growth is delayed somewhat. It is, however, not completely inhibited, and at the end of 48 hours necrosis and cytolysis of the explant ensues, the result of a very visible growth of bacteria. When the contaminated culture fluid was plated out on nutrient agar, a large number of colonies appeared in 48 hours. These were found to consist of gram-negative, motile rods and, on further test (lactose fermentation, indole formation, etc.), proved to belong to the *Escherichia coli* group. Any culture of amphibian tissues contaminated by such bacteria is doomed to rapid cytolysis.

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[1] Fischer, A., *J. Exp. Med.*, 1922, **36**, 393.

[2] Stöhr, P., Jr., *Arch. Entw. Mech.*, 1924, **102**, 426.

[3] Fell, H. B., *Proc. Roy. Soc. London*, Ser. B, 1934, **116**, 316; *Phil. Trans. Roy. Soc. London*, Ser. B, 1939, **229**, 407; *J. Roy. Microsc. Soc.*, 1940, **60**, 95.

[4] Holtfreter, J., *J. Exp. Zool.*, 1945, **98**, 161.

[5] Holtfreter, J., *J. Exp. Zool.*, 1943, **93**, 251.

It has been found that the addition of streptomycin in a concentration of 10 - 20 $\gamma$ per milliliter to the sulfadiazine bath of Detwiler *et al.* will render small amphibians and their tissues completely aseptic in 24 hours in over 95% of attempts. The technic used in this laboratory is as follows: A 0.5% (for embryos) or 1.0% (for larvae) solution of sodium sulfadiazine is made up in tap water. To this is added streptomycin in a concentration of 10-20 $\gamma$/ml. This solution is placed in sterile covered stender dishes, in each of which one or two embryos or larvae are placed. The animals are kept in this solution for 24 hours at the end of which time they are sterile and ready for operation. They are anaesthetized, if need be, in sterile MS 222.* The operative removal of the organ anlagen is carried out aseptically under a plexiglass shield mounted just below the oculars of a dissecting binocular microscope. Surgery is carried out using sterile iridectomy scissors, microscalpels, and watchmakers forceps. Each explant is removed from the operating dish with a mouth pipette, and placed in a bath of sterile Holtfreter solution. This bath is employed to promote the wound healing of the epidermis of the tissue piece, and to permit the diffusion from the tissue of the sulfadiazine and streptomycin solution. Explants are kept in these baths individually for a minimum of two hours. Following this, the tissue is removed from the bath with an absolute minimum of salt solution (again by mouth pipette) and transferred to a drop of nutrient on a sterile cover slip. The preparation is then sealed to a depression slide with a sterile paraffin-vaseline mixture. Experiments thus set up are placed in a constant temperature chamber at 20°C for further study.

The sulfadiazine-streptomycin solution here employed has exhibited no toxic effects whatever. Detwiler, Copenhaver and Robinson[6] reported a retardation of growth of their animals when kept in 0.5-1.0% sodium sulfadiazine. Such an effect has not been noticed in our experiments. Both larvae and embryos continue to grow at normal rates, to undergo morphogenesis and to differentiate in a normal manner. Larvae and embryos have been kept for as long as one week in this solution with no visible ill effects. Young embryos (neurulae) may be removed from their capsules in this solution and develop normally.

The procedure outlined above does not inhibit the growth of fungi which may contaminate amphibia. However, if precautions are taken in the maintenance of the animals in the laboratory, fungus infection can be kept at a minimum (approx. 2%).

2. *Culture medium.* The medium used in

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[6] Detwiler, S. R., Copenhaver, W. M., and Robinson, C. O., *J. Exp. Zool.*, 1947, **106**, 109.

* MS 222 $C_{10}H_{15}NO_5S$. A meta-amino-benzoic acid-ethylester in the form of a methan-sulfonate. (Sandoz Chemical Works, Basel and New York).

this study consisted of a mixture of two fluids: 1) Citrated rabbit plasma obtained by sterile heart puncture, and 2) a solution of half strength Holtfreter solution, containing as additions 0.25% agar, 0.045% sodium nucleate and 0.77% glucose. Equal portions of these two fluids were mixed to form the final medium. This medium has sustained the growth and differentiation of amphibian limb buds for as long as 20 days. Routinely, cultures are kept for from 4 to 12 days. For these periods it does not need to be replenished. This medium is not by any means considered to be perfect for amphibian tissue, nor is it felt that all requirements for growth and differentiation are supplied. It is hoped that further experiments will result in a medium in which all of the constituents are known chemically. The small amount of yolk included in embryonic explants undoubtedly constitutes a part of the essential nutrient. Tissues do survive, however, beyond the point where yolk is visible microscopically either in the culture or in the cells.

The above medium was developed after experiments had been made with the synthetic medium of White.[7] It was found that White's medium alone would not support the growth of embryonic or larval amphibian tissue. Also, the inorganic salt solution of White, when used in place of Holtfreter solution in the above medium, would not support growth.

3. *Epidermal closure of the wound.* It has been found that the piece of tissue in culture must be covered by the epibolically active epidermis before organogenesis will take place. Holtfreter solution, as a bath for the explant prior to placing it in the hanging drop, serves well to enhance the wound healing capacities of the epidermis. In earlier stages this process may be observed under the low power microscope. When healing of the epidermis does not take place, the explant forms a culture characterized by migrating cells and loss of structural organization. The medium as used also favors epithelial permanence, *i.e.*, if the tissue is not in contact with the glass cover slip, but rather hangs down in the center of the drop. Epiboly of the epidermis is not hindered by the contact of the explant with the medium-air interface in the well of the slide. The agar contained in the medium tends to increase its viscosity to the consistency of a soft mush (Spratt[8]). The agar, plus the lack of fibrin strands to promote the outwandering of cells, is thought to assist in the maintenance and development of morphological organization of the explant within its epithelial sac.

By the use of the technics described above, it has been possible to study the differentiation *in vitro* of such complex organs and organ systems as limbs, hearts, gills, balancers, and eyes. Differentiation has been, in all cases where it occurred, essentially normal, although somewhat slower than in the intact controls.

*Summary.* 1. A technic is described for rendering surface and subsurface tissues of amphibia wholly sterile for use in tissue culture. Advantage is taken of the nontoxic selective antibiotic action of small concentrations of sodium sulfadiazine and streptomycin.

2. A medium which supports organogenesis, differentiation and growth of isolated organ and organ system primordia of amphibian embryos has been reported. The principal components of this medium are citrated plasma, salts, agar, glucose and sodium nucleate.

3. Morphogenetic activity in an explanted piece of tissue occurs only when the epidermis heals so as completely to cover the explant

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[7] White, P. R., *Growth*, 1946, **10**, 231.

[8] Spratt, N. T., Jr., *J. Exp. Zool.*, 1947, **10** 345.

## 16727

# Value of Urea in the Diet of Rabbits.

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The rabbit shows some very interesting peculiarities in its nutrition in that it does not require a dietary source of riboflavin, pantothenic acid, biotin or folic acid.[1] On the basis of the excretion of these vitamins it is apparent that there is considerable microbial synthesis in the digestive tract of the rabbit. Rabbits show a normal functional coprophagy which presumably enables them to make better utilization of the vitamins synthesized in the digestive tract. It has been shown that more than 50% of the feces produced by the rabbit is normally reingested.[2]

The literature on the synthesis of the B vitamins and of protein by microorganisms in the paunch of ruminants has been previously reviewed.[3-5] It is well established that elemental forms of nitrogen such as urea can be converted into protein by microorganisms in the rumen of polygastric animals and that the protein is utilized by the host animal. The fact that the rabbit has a large cecum favorable to microorganisms and the recent observations showing that they do not require a dietary source of some of the B vitamins prompted us to investigate the possibility of amino acid synthesis by the rabbit and its utilization during the normal process of feces ingestion. Observations have also been made in the course of these studies on the effect of decreasing the level of protein on the requirements for vitamin A.

*Experimental.* Weanling rabbits, 8 weeks old, of the New Zealand White breed and weighing 800 g to 1200 g were used in these studies. There were 5 rabbits in each group with an equal number of males and females. These were kept in wire bottom cages. This type of cage does not prevent coprophagy in the rabbit. Group I was fed a diet composed of purified casein (Labco) 10%, *DL*-methionine 0.30%, cerelose 68.2%, cellulose 10%, Salts mixture[6] 3%, corn oil 8%, A & D Oil (Nopco XX) 0.5%. The following amounts of vitamins were added per 100 g of diet: mixed tocopherols 50 mg, choline chloride 200 mg, niacin 20 mg, inositol 10 mg, pyridoxine hydrochloride 0.7 mg, thiamine hydrochloride 0.7 mg, riboflavin 0.7 mg, calcium pantothenate 1 mg, and 2-methyl, 1,4-naphthoquinone 0.075 mg. Group 2 was fed a diet similar to the above except that 3.43% of urea was added at the expense of an equal amount of dextrose to make the protein equivalent (N x 6.25) to 20%. The third group received 20% of casein and no methionine. At the end of the 4th week the diets were slightly modified by replacing wood pulp for the cellophane as a source of cellulose. The methionine was added at levels so that the methionine content of the diets containing 10% of casein was equivalent to that of the diet containing 20% of casein. Since methionine has been shown to favor the synthesis of amino acids in ruminants[7] it was added to eliminate this as a factor of variation with regards to the control group receiving 20% casein. Water and food were given *ad libitum*. The rabbits were kept on experiment for 10 weeks and weighed at weekly intervals.

The average growth response of the animals in each group is shown in Fig. I. As can be

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[1] Olcese, Orlando, Pearson, P. B., and Schweigert, B. S., *J. Nutrition*, 1948, **38**, 577.

[2] Eden, A., *Nature*, 1940, **145**, 36.

[3] Goss, Harold, *Nutrition Abst. and Rev.*, 1943, **12**, 531.

[4] McNaught, M. L., and Smith, J. B. A., *Nutrition Abstr. and Rev.*, 1947, **17**, 18.

[5] Kon, S. K., and Porter, J. W. G., *Nutrition Abstr. and Rev.*, 1947, **17**, 31.

[6] Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, **138**, 459.

[7] Loosli, J. K., and Harris, L. E., *J. Anim. Sci.*, 1945, **4**, 435.

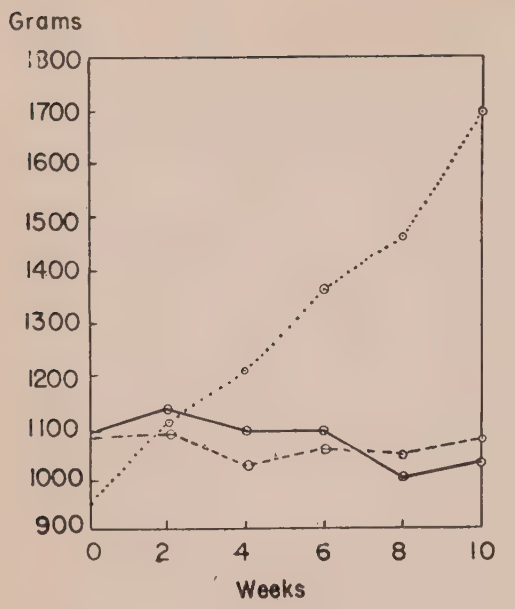

FIG. 1.

Growth of rabbits on diet containing 20% casein is indicated by dotted line. Broken line shows weight of rabbits receiving diet containing 10% casein and solid line weight of rabbits fed diet containing 10% casein plus 3.43% urea.

readily seen the group receiving 20% casein was the only group that made satisfactory gains. The groups receiving either the 10% casein alone or 10% casein plus 3.43% urea both lost weight. Since the rabbits receiving urea as a supplement to a low protein diet made no more gain than those on 10% of casein it is apparent that there is no significant synthesis of protein by microorganisms in the gastrointestinal tract of the rabbit.

At the end of the 3rd week 3 rabbits in each of the groups on the low protein diets showed some eye lesions. The main symptom was a conjunctivitis. A viscid conjunctival secretion was present and the lids were stuck together. Usually these symptoms became noticeable in both eyes at the same time. Oral administration of A and D oil for a period of one week relieved the animals of these early symptoms of vitamin A deficiency but when the administration was stopped the symptoms reappeared in one week. Again the A and D oil was administered for 1 week and the symptoms disappeared but they reappeared as soon as the administration of the and D oil was discontinued on the 7th week. None of the rabbits on the diet containing 20% casein showed vitamin A deficiency symptoms. Since the administration of the extra vitamin A did not influence the growth of the rabbits it may be assumed that the failure of the rabbits on the low protein diets to grow was due to a deficiency of protein *per se.* The fact that a vitamin A deficiency occurred with the rabbits fed low protein diets is very significant and suggests an interrelationship in the diet between proteins and fat soluble factors. This may be related to the preservation of vitamin A.

Although no carefully controlled experiments have been reported on vitamin A deficiency produced by the partial replacement of protein concentrates by urea, some contradictory evidence exists in the literature with regards to the effect of adding urea to silage. While Cullison[8] has reported an increased content of carotene in silage which had received urea supplementation, Wise, *et al.*[9] found losses of carotene in silage that was fed with urea as a supplement. On the other hand, Woodward and Shepherd[10] have found no appreciable differences in the carotene content of silage containing urea and silage not containing urea.

Destruction of vitamin A can not be attributed to the addition of urea to the diet low in protein, because, although the rabbits fed this diet developed vitamin A deficiency symptoms, similar symptoms were shown by the rabbits fed the low protein diet that did not contain urea. The ill effects are therefore attributable to the lower protein level in the diet. Since it is very doubtful that the lower protein intake interferes with the absorption of fat-soluble factors, the mechanism by which the additional protein produces its beneficial effects is probably related to the synergistic effect of the amino acids in the stabilization of the fat. Clausen, Lundberg and Burr[11] have shown that by the addition of adequate amino acid synergists the effectiveness of natural anti-oxidants, which are present in the fats in appreciable quantities, may be greatly increased. This stabilization of the fat may be an important factor in reducing vitamin A and carotene destruction.

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[8] Cullison, A. E., *J. Anim. Sci.*, 1944, **3**, 59.

[9] Wise, G. H., Mitchell, J. H., La Master, J. P. and Roderick, D. B., *J. Dairy Sci.*, 1944, **27**, 649.

[10] Woodward, T. E., and Shepherd, J. B., *J. Dairy Sci.*, 1944, **27**, 648.

[11] Clausen, D. F., Lundberg, W. O., and Burr, G. O., *J. Am. Oil Chem. Soc.*, 1947, **24**, 403.

*Summary.* The addition of urea to a diet low in protein did not increase the rate of gain of rabbits. From the data it is apparent that this species cannot use urea in the diet as a replacement of part of the protein. Rabbits fed diets containing 10% of casein as the sole source of protein or 10% casein plus 3.43% urea developed typical vitamin A deficiency symptoms as compared with the control group fed a diet similar except that it contained 20% of casein. The results suggest that protein has a protective action on the oxidation of vitamin A in the diets or that it has a sparing action on the vitamin A requirements of the rabbit.

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## 16728

# Pantothenic Acid and the Metabolism of Amino Acids by Bacteria.*

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Dorfman, Berkman and Koser,[1] and Hills[2] first observed that pantothenic acid functions in the pyruvic acid metabolism of *Proteus morganii.* More recently, Lipmann, *et al.*,[3] and Novelli and Lipmann[4] have found that pantothenate is a part of a coenzyme which is concerned with an acetylation system in tissues and with the breakdown of pyruvate to acetate and other products. They isolated this coenzyme, which they termed "coenzyme A", and were able to recover pantothenic acid from it. They also found that the amount of pantothenate bound in the cells closely correlated with the increased stimulation of pyruvate oxidation, and devised a method for the assay of coenzyme A.[5]

The present study was undertaken to determine if pantothenic acid has any effect on the metabolism of amino acids or other related compounds by bacteria.

*Technic.* The chemically-defined medium employed in this study for growing the bacteria has already been described.[6] The organism used was *Proteus morganii.* Pantothenate deficient cells were prepared as follows: A small amount of growth was removed from a 6-hour agar slant culture by means of a sterile needle and cultured in 10 ml of a chemically-defined medium for 24 hours at 37°C. One ml of this culture was then used to inoculate larger quantities of the same basal medium containing in this case only 0.01 $\mu$g of Ca-pantothenate per ml. The "pantothenate-deficient" cells from 4 liters of a 24-hour culture were then harvested by means of a Sharples centrifuge, and washed twice with 50 ml of sterile saline by the ordinary centrifuge technics. From this quantity of medium it was possible to obtain yields of cells ranging in dry weight from 150

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* This study was supported in part by a grant from the Williams-Waterman Fund for the combat of dietary diseases. Certain of the vitamins and amino acids were kindly supplied by Merck & Co.

[1] Dorfman, A., Berkman, S., and Koser, S. A., *J. Biol. Chem.*, 1942, **144**, 393.

[2] Hills, G. M., *Biochem. J.*, 1943, **37**, 418.

[3] Lipmann, F., Kaplan, N. O., Novelli, G. D., Tuttle, L. C., and Guirard, B. M., *J. Biol. Chem.*, 1947, **167**, 869.

[4] Novelli, G. D., and Lipmann, F., *Arch. Biochem.*, 1947, **14**, 23.

[5] Kaplan, N. O., and Lipmann, F., *J. Biol. Chem.*, 1948, **174**, 37.

[6] Pelczar, M. J., Jr., and Porter, J. R., *J. Biol. Chem.*, 1941, **139**, 111.

TABLE I.
Effect of Pantothenate on Ammonia Liberation and on Oxidation of Amino Acids by *Proteus morganii.*

| Amino acid | μg $NH_3$ produced in 90 min.* | | μl $O_2$-uptake in 90 min.* | |
|---|---|---|---|---|
| | Without pantothenate | With pantothenate | Without pantothenate | With pantothenate |
| Glycine | 10 | 12 | 3 | 5 |
| dl-Alanine | 13 | 23 | 7 | 10 |
| dl Valine | 7 | 16 | 22 | 26 |
| dl-Threonine | 30 | 43 | 8 | 17 |
| dl-Phenylalanine | 5 | 8 | 0 | 5 |
| dl-Methionine | 14 | 14 | 0 | 6 |
| dl-Isoleucine | 13 | 20 | 25 | 29 |
| dl-Citrulline | 0 | 2 | 13 | 15 |
| l-Tryptophane | 4 | 6 | 0 | 0 |
| l-Proline | 5 | 5 | 3 | 5 |
| dl-Norleucine | 20 | 25 | 6 | 13 |
| dl-Lysine. HCl | 2 | 4 | 6 | 8 |
| l-Histidine. HCl | 4 | 6 | 0 | 5 |
| l-Cystine | 11 | 15 | 15 | 25 |
| d-Arginine. HCl | 5 | 8 | 13 | 19 |
| dl-Tyrosine | 8 | 14 | 8 | 10 |
| dl-Serine | 74 | 79 | 85 | 94 |
| dl-Norvaline | 27 | 35 | 13 | 14 |
| l-Hydroxyproline | 0 | 0 | 0 | 4 |
| dl-Glutamic acid | 20 | 48 | 23 | 172 |
| dl-Aspartic acid | 16 | 30 | 12 | 50 |
| dl-Leucine | 30 | 38 | 12 | 13 |

* Average values for several determinations; all data corrected for controls which were 3-5 μg of $NH_3$ and 10-15 μl $O_2$.

to 200 mg. After the final washing and centrifugation the cells were resuspended in saline to give a final concentration of 10 mg of cells per ml. The substrates employed in this study consisted of the 22 amino acids and certain organic acids shown in the tables. These compounds were all prepared in concentrated aqueous solutions, and used as M/450 final concentrations.

For the deamination studies the micro-diffusion method of Conway[7] was employed, and the ammonia determinations were made as follows: The substrates (amino acids) plus pantothenate were added to the outside well of the diffusion cell, together with phosphate buffer (pH 8.0) and a 5 mg suspension of bacteria, to give a final quantity of 1.5 ml. One ml of N/150 HCl, containing an indicator, was placed in the center cup of the diffusion cell. Following an incubation period of 90 minutes at 37°C, 1 ml of saturated $K_2CO_3$ solution was added to the outside well to release the ammonia, and the units were again incubated for one hour. The acid in the center cup was then titrated with N/75 NaOH, and the amount of ammonia liberated from the amino acids calculated.

For the respiration studies, standard manometric methods were employed. The substrates were added so as to give M/450 final concentrations; 0.1 ml of N/10 HCl was placed in the side bulb to absorb any ammonia given off by the 1.0 ml bacterial cell suspensions; and 0.3 ml of a 20% KOH solution was placed in the center well of the Warburg vessels. In every case the final volume of the vessels was brought to 3.0 ml with phosphate buffer (pH 7.3). The flasks were then equilibrated for 10 minutes in the water bath before readings were started.

*Experimental and results.* From the data in Table I it will be seen that serine was the most readily deaminated amino acid. However, since this reaction was not stimulated by pantothenate it does not appear to be involved in the process. Alanine, threonine, norleucine, norvaline, glutamic acid, aspartic acid, and leucine were also deaminated in varying degrees, and in certain of these cases the presence of pantothenate appeared to stimulate the process. This was especially true in the case of glutamic and aspartic acids. Few of the other amino acids were attacked by this organism, nor did the presence of pantothenate stimulate ammonia liberation.

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[7] Conway, E. J., *Micro-diffusion Methods and Volumetric Error*, 1940, D. Van Nostrand Co., New York.

Since it is generally accepted that deamination is one of the first steps in the breakdown of amino acids, especially at the pH values where the above experiments were conducted, an attempt was made to determine the role played by pantothenate in the metabolism of certain of these substances. It was found after a few preliminary experiments that the respiration of "pantothenate-deficient" cells of *Proteus morganii*, using amino acids as substrates, showed a definite lag phase during the first half hour or so of the experiment. Since it has been reported by McIlwain[8] that a time factor is involved when deficient cells bind pantothenate, it was decided to divide the cell suspensions into two equal portions after the final washing, and add 1.0 μg per ml of pantothenate to one portion of the cells. When the cells were treated in this manner, followed by incubation at 37°C for about 2 hours, little or no lag was noted in their early respiration period.

From the oxygen uptake values (Table I) it will be seen that serine gave the highest values for deficient cells and likewise quite high values for non-deficient cells. As in the case of the deamination studies, however, pantothenate did not stimulate the oxidation of serine. On the other hand, when such amino acids as threonine, norleucine, cystine, and especially glutamic and aspartic acids were used as substrates, pantothenate did stimulate their oxidation in varying degrees. The fact that the oxidation of these amino acids is stimulated by pantothenate would seem to indicate that pyruvate, which is known to be stimulated, is the key intermediate in their metabolism. With this in mind glutamic acid and aspartic acid were chosen as representative amino acids for further metabolic studies. In these experiments glutamic acid and aspartic acid were studied together with their known, or postulated, intermediates in an attempt to discover at which point, or points, in their metabolism pantothenic acid functions. The intermediates studied were α-ketoglutarate, succinate, fumarate, oxalacetate and pyruvate, and these were kept on an equal molar basis

TABLE II.
Effect of Pantothenate on Oxidation of Glutamic Acid, Aspartic Acid, and Their Metabolic Intermediates.

| | μl $O_2$ uptake in 90 min.* | |
|---|---|---|
| | Without pantothenate | With pantothenate |
| Glutamic acid | 20 | 170 |
| α-Ketoglutarate | 16 | 143 |
| Succinate | 44 | 103 |
| Fumarate | 10 | 23 |
| Oxalacetate | 29 | 55 |
| Pyruvate | 30 | 64 |
| Aspartic acid | 15 | 55 |

* Average values for 4 determinations; all data corrected for controls which gave 5-15 μl $O_2$ uptake.

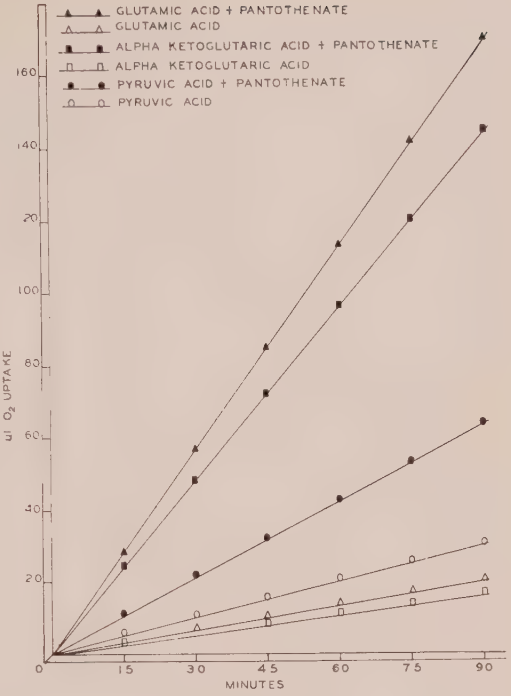

FIG. 1.
Effect of pantothenate on oxidation of glutamic acid, α-ketoglutarate, and pyruvate.

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[8] McIlwain, H., *Biochem. J.*, 1945, **39**, 279.

with glutamic acid. The results of these studies are shown in Table II and Fig. 1. From these data it can be seen that pantothenate stimulated respiration considerably when either α-ketoglutarate or glutamic acid was used as a substrate, but succinate, fumarate, oxalacetate and aspartic acid were oxidized to lesser extent. It will be noted that the stimulation values for most of these substrates are in the same range as was observed with pyruvate. It was also possible to demonstrate pyruvate in the vessels after all of these substrates had been oxidized. Thus it can probably be assumed that pantothenate stimulation of certain amino acids and other substrates is due, in part at least, to their being converted to pyruvate which is in turn stimulated. However, the pantothenate stimulation observed when glutamic acid or α-ketoglutarate was used as substrate cannot be accounted for entirely as being due to pyruvate stimulation alone. Therefore it appears that pantothenic acid also functions in some other way in the oxidation of these substrates. Since the values observed with glutamic acid were very close to those noted with α-ketoglutarate, it would seem to eliminate the possibility of pantothenic acid functioning in the deamination of glutamic acid.

*Summary.* From this study is can be concluded that the pantothenate stimulation of amino acid oxidation by *Proteus morganii* is limited to those amino acids which can be converted to pyruvate. However, in the case of glutamic acid, and its metabolic intermediate α-ketoglutarate, the pantothenate stimulation appears to be more than can be attributed to pyruvate oxidation.

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## 16729

# Testicular Degeneration as a Result of Microwave Irradiation.

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The mammalian scrotum has been established as being a local thermoregulator for the testes. Moore and Quick[1] found the scrotal temperature of white rats to be from 2° to 8°C lower than the temperature of the abdominal cavity. A sub-abdominal temperature in the scrotum has been shown to be necessary for the continuance of spermatogenesis. Moore[2] confined the testes of guinea pigs in the abdominal cavity for varying periods of time and found that an abdominal retention of seven days resulted in a complete disorganization of the germinal epithelium of the seminiferous tubules. He considered the cause of this degeneration to be due to the higher temperature of the abdominal cavity.

Investigations of Moore[3] showed that testicular degeneration resulted from a single application of heat at approximately 7°C above body temperature for a 15-minute period. The heating devices which were used consisted of hot water baths, electric stoves, electric light bulbs, and hot water pads. Testicular degeneration was visible histologically within four to six days following the heat application, and was entirely similar in type to that resulting from early experimental cryptorchidism.

Fukui[4] exposed the scrotum of rabbits to sunlight and to warm air, and found a definite relationship between the temperature and the time required to cause regressive changes in the germinal cells. The minimum scrotal temperature at which he was able to produce testicular damage was 40°C. The time of required exposure at this temperature was more than one hundred hours.

It was the purpose of this study to determine the effect of 12 cm electromagnetic radiations of testicular tissue. In addition, attempts were made to confirm the results of previous investigators relative to the effects of infra red irradiations on testes.

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[1] Moore, C. R., and Quick, W. J., *Am. J. Physiol.*, 1924, **68**, 70.

[2] Moore, C. R., *Am. J. Anat.*, 1924, **34**, 269.

[3] Moore, C. R., *Am. J. Anat.*, 1924, **34**, 337.

[4] Fukui, N., *Japan M. World*, 1923, **3**, 27.

*Procedure.* Male albino rats of the Sprague-Dawley strain, ranging in age from 120 to 200 days were employed in this study. In preparation for irradiation the animals were anesthetized with ether and the scrotum swabbed with 95% alcohol. Each animal was then arranged on a platform behind a copper shield, and the scrotum was inserted through an opening provided in the shield. Thus, the remainder of the animal was protected from the radiations. An iron-constantan thermocouple needle, of the type described by Tuttle and Janney,[5] was then inserted into the center of one of the testes. This served to register the degree of temperature produced. The thermocouple had been calibrated previously with a Bureau of Standards thermometer. Thermocouple potentials were measured by a Leeds and Northrup potentiometer. Temperatures were read to the nearest tenth of a degree centigrade. The temperature of the testes was allowed to fall to approximately 29°C before the irradiations were started. Because of possible damage to the testes resulting from thermocouple needle puncture, one testis was employed for temperature measurements and the contralateral testis was used to study the histologic effects of the radiation. Care was taken to align both testes at an equal distance from the source of radiation. In preliminary experiments it was found that if these precautions were taken the temperature rise due to the radiations was the same in both testes.

A Raytheon Microtherm generator (model CMD4) which produced a wave length of approximately 12 cm was used to apply the high frequency radiations. A variac was provided by means of which the power output was regulated. The corner type reflector was used. The infra-red source rated at 600 watts was of the non-luminous type, and a 9-inch hemispherical reflector was employed. In all cases irradiation was applied to the testis through the scrotum.

Two series of experiments were performed with 12 cm electromagnetic waves. In the first of these series the procedure consisted of elevating the testicular temperature to levels of 47, 46, 45, 44, 43, 40, 37, 35, 34, 33, 32, 31 and 30°C. For each temperature level 4 animals were used, and the testicular temperature was maintained at the selected temperature for a single period of 5 minutes. The temperature was maintained by varying the power output of the microwave machine. From each group of 4, an animal was sacrificed at 4, 8, 12 and 16 days following the exposure and the testes were prepared for histologic examination.

In the second series of irradiations 2 groups of 10 animals were given single 15-minute exposures at temperature levels of 33° and 34°C. A third group of 10 animals was exposed to 35° C for a period of 10 minutes. All animals were sacrificed for histologic studies 4 days following the exposures. The testes were fixed in Bouin's solution and the sections stained in hematoxylin-eosin.

Infra-red irradiation was used in two series of experiments. In the first series testicular temperatures were raised by irradiation to levels of 33, 37, 40, and 43°C for periods of 5 minutes. Four animals were exposed at each temperature level and a histologic examination of the testes was made at 4, 8, 12, and 16 days after the exposures. The procedure in the second series of infra-red irradiations was to expose two groups of 10 animals to temperature levels of 38° and 40°C for 10 minutes. Four days after exposure the animals were sacrificed and the testes were removed for histologic study.

The testes of 12 normal non-irradiated males were employed as histologic controls.

*Results.* The results of experiments with 12 cm electromagnetic irradiations are listed in Table I. These results show that in all cases when the testicular temperature was raised to 35°C or higher there was evidence of testis tissue damage. At temperatures from 31° to 35°C approximately 50% of the testes showed signs of degenerative changes. The

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[5] Tuttle, W. W., and Janney, C. D., *Arch. Phys. Med.*, 1948, **29**, 416.

TABLE I.
Effect of 12 Cm Irradiation on Male Gonads.

| No. animals exposed | Exposure temp. °C | Time of exposure min. | Animals with testicular damage | % with testicular damage |
|---|---|---|---|---|
| 4 | 47 | 5 | 4 | 100 |
| 4 | 46 | 5 | 4 | 100 |
| 4 | 45 | 5 | 4 | 100 |
| 4 | 44 | 5 | 4 | 100 |
| 4 | 43 | 5 | 4 | 100 |
| 4 | 40 | 5 | 4 | 100 |
| 4 | 37 | 5 | 4 | 100 |
| 4 | 35 | 5 | 4 | 100 |
| 4 | 33 | 5 | 2 | 50 |
| 4 | 32 | 5 | 1 | 25 |
| 4 | 31 | 5 | 1 | 25 |
| 4 | 30 | 5 | 0 | 0 |
| 13 | 35 | 10 | 13 | 100 |
| 4 | 33 | 10 | 1 | 25 |
| 4 | 31 | 10 | 1 | 25 |
| 3 | 35 | 15 | 3 | 100 |
| 7 | 34 | 15 | 3 | 43 |
| 16 | 33 | 15 | 9 | 56 |
| 4 | 31 | 15 | 0 | 0 |

TABLE II.
Effect of Infra Red Irradiation on Male Gonads.

| No. animals exposed | Exposure temp. °C | Time of exposure min. | Animals having testicular damage | % with testicular damage |
|---|---|---|---|---|
| 3 | 43 | 5 | 2 | 67 |
| 4 | 40 | 5 | 2 | 50 |
| 4 | 37 | 5 | 0 | 0 |
| 4 | 33 | 5 | 0 | 0 |
| 8 | 40 | 10 | 3 | 38 |
| 10 | 38 | 10 | 0 | 0 |

testes of animals exposed to 30°C were not affected.

The results of experiments with infra red irradiation (Table II) show that testes from 67% of the animals exposed to 43°C were damaged. Testicular degeneration was not found in the experiments where the temperature of the testes was maintained at 38°C for 10 minutes by infra red. No damage was found in the testes of control animals.

A typical histological picture of testicular degeneration following exposure to electromagnetic waves shows an area of degeneration along the side of the testis nearest to the source of radiation (area A, Fig.1). Continuing from this area of degeneration to the opposite side of the testis, all gradations from completely degenerated to normal tubules can be found (areas B and C, Fig. 1). Fig. 2 clearly shows the transition of degeneration from area A to area B of Fig. 1. These areas are well defined. A detailed picture of a degenerating tubule in area B is shown in Fig. 3. In area A, which was nearest to the source of radiation, complete coagulation of the tubules was found (area A of Fig. 2). This area showed an absence of germ cell nuclear material, the cytoplasm appearing much more refractile than normal cytoplasm. The tissue resembled that seen in burn necrosis. In general the tubules in area B show sloughing of degenerating germinal elements into the lumen, multinuclear masses termed "giant cells" which apparently consist of fused spermatid nuclei, and usually absence of spermatozoa (area B of Fig. 2 and 3). Sertoli and interstitial cells apparently remain intact. Leucocytic infiltration of the

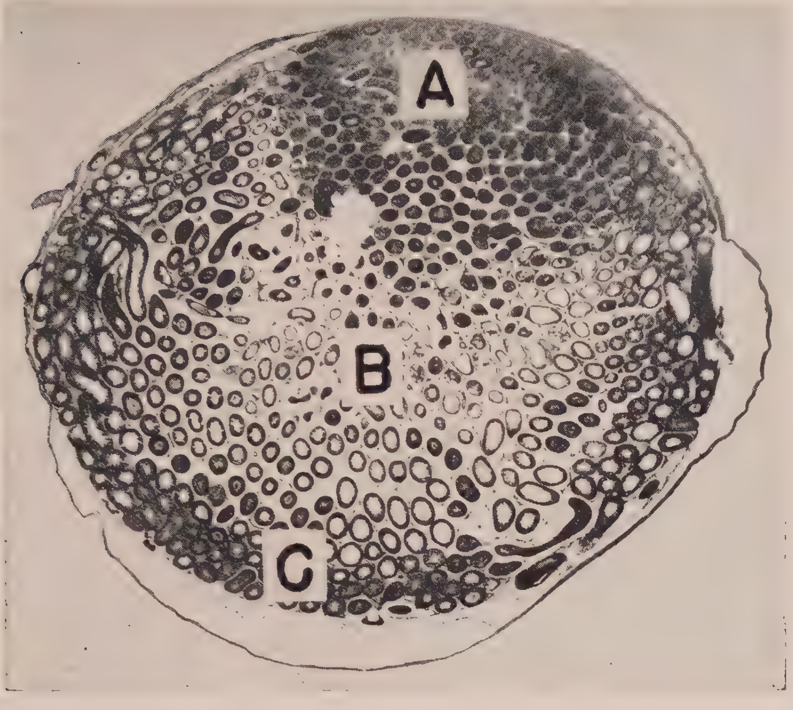

FIG. 1.
Cross-section of entire testis removed 4 days after a single irradiation with 12 cm microwaves at 35°C for 10 minutes (× 10). See text for explanation of the areas indicated.

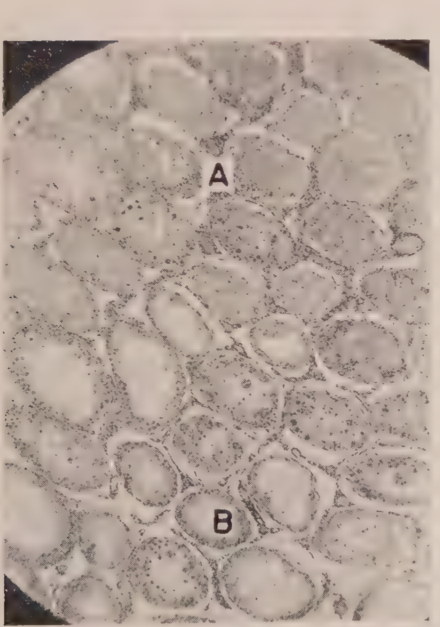

FIG. 2.
Seminiferous tubules from areas A and B of Fig. 1. (× 40).

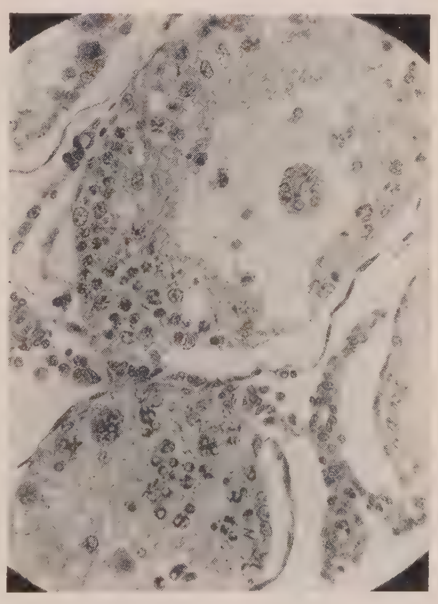

FIG. 3.
High magnification of a degenerating seminiferous tubule from area B of Fig. 2. (× 200).

tissue was evidenced by the presence in some cases of intertubular polymorphonuclear leucocytes, in the areas of degeneration. In area C of Fig. 1, the tubules were essentially normal.

Fig. 4 is a cross section through the center of a testis exposed for 10 minutes at 40° C to infra red irradiations. Area A, the side most directly exposed, shows coagulation of the tubules, whereas the tubules in area B show varying degrees of degeneration. The tubules farthest from the source of radiation were normal. The general picture of the degenerative changes was similar to that produced by microwave irradiation.

*Discussion.* Testicular degeneration resulting from exposures to microwaves and infra red irradiations presented a similar histologic appearance which was typical of the degeneration seen in experimental cryptorchidism.

The temperatures at which damage was noted from infra red irradiations were approximately 3 to 5°C lower than those reported by Moore and Chase. These investigators placed the bulb of a thermometer close to the scrotum to register the degree of heat applied. With the needle thermocouple method of temperature measurement used in the experiments reported here it was possible to register the temperature within the testes.

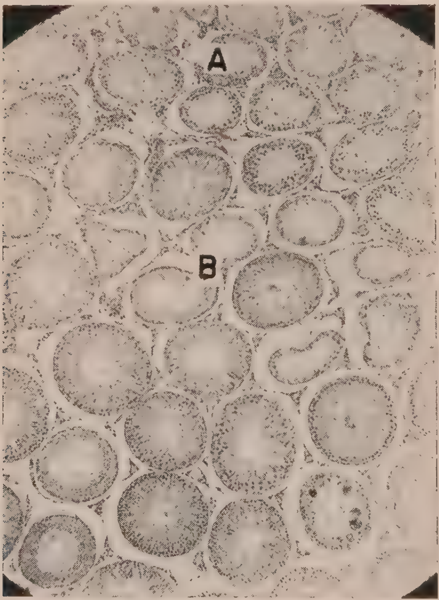

FIG. 4.
Degenerating tubules of a testis 4 days after a single irradiation with infra red at 40°C for 10 minutes. (× 40).

Following electromagnetic irradiation testicular degeneration was found at temperatures below those at which damage occurred from infra red irradiations. All of the testes which were elevated to a temperature level of 35°C and above with microwaves were found to contain degenerated tubules.

The outcome of this experiment clearly shows that testicular damage will result from 12 cm irradiations at a temperature below that of the abdominal cavity and below that necessary to cause injury by infra red exposures. This finding suggests that damage may result in part from factors other than heat. However, it should be pointed out that measurements of temperature were made only near the center of the testes and the possibility exists that areas adjacent to the field of irradiation may have been subjected to temperatures somewhat in excess of those recorded.

These findings suggest that precautions should be taken by those working in the field of high frequency electromagnetic generators and to those giving treatments with microwave generators. Because of the unusual susceptibility of testicular tissue to thermal agents, it seems desirable to shield these structures from high frequency electromagnetic waves during periods of treatment or exposure.

*Summary.* A study was made concerning the effects of 12 cm electromagnetic waves and of infra red irradiations upon the testes of adult albino rats. A single ten-minute exposure to microwaves at a temperature of 35°C as measured in the central areas of the testes caused testicular degeneration in all cases. In some experiments testicular damage resulted from a single exposure at temperatures between 30° and 35°C. Testicular damage was not found in experiments in which infra red irradiation was applied for 10 minutes at 38°C but was observed when applied at a temperature of 40° C and above. The type of degeneration resulting from microwave exposure could not be distinguished from that produced by infra red.

### 16730

## Intravenous Infusions of a Combined Fat Emulsion into Dogs.*

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The availability of an intravenous emulsion of fat, protein and glucose for experimental and clinical investigation has opened new fields of study in parenteral nutrition.[1] A combined emulsion such as developed in our laboratory was administered intravenously with equal facility to human patients[2] as well as to animals with a favorably low incidence of infusion reactions. The present report is devoted to observations made on a series of dog recipients of the combined emulsion.

*Method of study.* Nineteen dogs were divided into 3 groups: Group I, seven dogs received single infusions of the combined emulsion in a variety of acute experiments. Group II, six dogs were maintained on daily infusions of the emulsion and a standard kennel diet. Group III, six dogs were carried exclusively on the intravenous emulsion as the sole nutritive source.

Two types of combined emulsion were used. The first type contained 5% coconut oil, 5% amigen and 5% glucose solution emulsified with a 6% special intravenous gelatin solution. The second type of combined emulsion contained 10% coconut oil with the remaining ingredients at the original concentrations. The caloric value of the 5% emulsion was 0.85 calories per ml, that of the 10% emulsion was 1.3 calories per ml. The emulsion was prepared in a special homogenizer constructed to our specifications so that the entire process of blending and homogenization could be carried out under sterile conditions and the resultant yield collected in sterile bottles. The use of the autoclave as a final step in the procedure was obviated. The process of manufacture and a description of the homogenizing machine were described elsewhere in greater detail.[1,2]

The following laboratory tests were made, the number of tests performed depending on the experiment; hemoglobin (Sahli), red blood cell count, white blood cell count, refractive index of plasma (Fischer refractometer), specific gravity of blood and plasma (Scudder apparatus) chylomicra count,[3] total serum lipids and serum lipase.[4] The weight of the animals was recorded at regular intervals. After the completion of an experiment the dog was sacrificed and selected tissues were stained with special fat dyes for microscopic study.

*Results.* Group I: Three dogs were anesthetized with sodium pentobarbital, the femoral artery was cannulized and connected to a recording manometer. The blood pressure was continuously recorded throughout the infusion and for 2 hours thereafter. In all three of the dogs there was an elevation of 15 to 18% in blood pressure above the initial level. This occurred after ¾ of the total amount of emulsion had been infused. In 2 dogs the thoracic duct was intubated with a Lindemann needle and samples of lymph were tapped at regular intervals. The flow of lymph increased about 22% during the infusion period. The specific gravity of the lymph and refractometric examinations showed little change indicative of the presence or appearance of the emulsion into the lymph circulation. In each of 2 dogs the effect of rapid and slow infusion of the emulsion was tested.

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* This project was made possible by grants from the Ben Lewis Fund in Experimental Surgery.

[1] Shafiroff, B. G. P., and Frank, C., *Science*, 1947, **106**, 474.

[2] Unpublished.

[3] Frazer, A. C., and Stewart, H. C., *J. Physiol.*, 1939, **95**, 21.

[4] Lagerlof, H., *Acta med. scandinav.*, 1945, **102**, 407.

TABLE I.
Effect of Infusions of Combined Emulsion.

| Dog No. | Group | Days | Total g fat infused | Wt. (kg) | Hemoglobin (g %) | Red blood cell count (million) | Hematocrit (Wintrobe) | Plasma protein (g %) |
|---|---|---|---|---|---|---|---|---|
| 9 | II—5% combined emulsion 250 ml + diet | 1 | 12.5 | 14.3 | 12.9 | 5.73 | 45 | 5.62 |
| | | 7 | 87.5 | 14.3 | 12.9 | 6.62 | 45 | 5.80 |
| | | 15 | 187.5 | 15.0 | 13.1 | 5.62 | 43 | 5.78 |
| | | 21 | 262.5 | 15.6 | 13.9 | 6.50 | 45 | 6.21 |
| | | 30 | 375 | 15.8 | 13.9 | 6.28 | 49 | 6.17 |
| 11 | II—5% combined emulsion 500 ml + diet | 1 | 25 | 11.0 | 10.2 | 4.52 | 51 | 4.96 |
| | | 7 | 175 | 11.0 | 12.9 | 5.81 | 49 | 5.48 |
| | | 15 | 375 | 11.2 | 12.2 | 6.26 | 44 | 5.23 |
| | | 21 | 525 | 11.3 | 13.2 | 6.08 | 48 | 5.44 |
| | | 30 | 750 | 11.8 | 12.9 | 6.00 | 49 | 5.35 |
| 12 | II—5% combined emulsion 500 ml + diet | 1 | 50 | 16.8 | 15.3 | 5.90 | 51 | 6.26 |
| | | 8 | 400 | 16.3 | 15.0 | 5.80 | 51 | 6.00 |
| | | 15 | 750 | 16.6 | 15.0 | 5.84 | 50 | 6.31 |
| 14 | III—10% combined emulsion no food | 1 | 50 | 16.5 | 14.5 | 6.18 | 49 | 5.92 |
| | | 8 | 400 | 16.0 | 14.9 | 6.20 | 50 | 5.92 |
| | | 15 | 750 | 15.5 | 15.2 | 6.10 | 48 | 6.00 |
| 19 | III—10% combined emulsion no food | 1 | 50 | 17.0 | 15.5 | 6.62 | 52 | 6.78 |
| | | 8 | 400 | 17.2 | 16.0 | 6.58 | 51 | 6.90 |
| | | 15 | 750 | 17.0 | 15.2 | 6.25 | 53 | 6.62 |

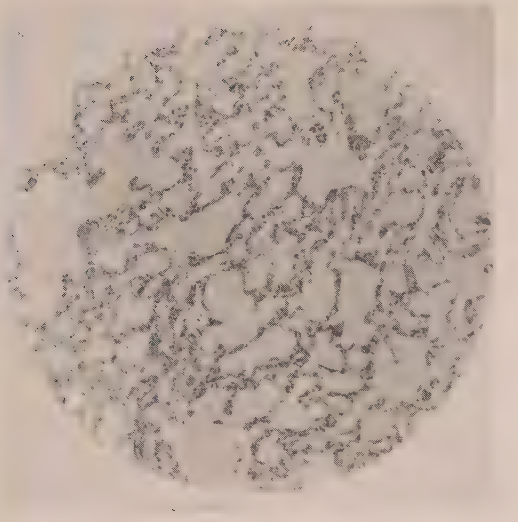


FIG. 1.

Section through the lung of a dog from Group III maintained exclusively on the 10% combined emulsion for a period of 14 days. The lung is microscopically normal.

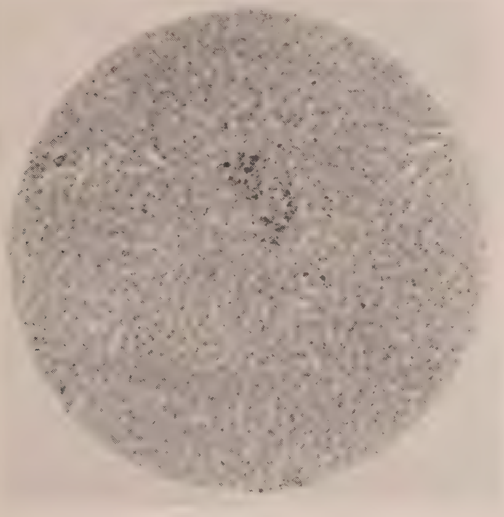
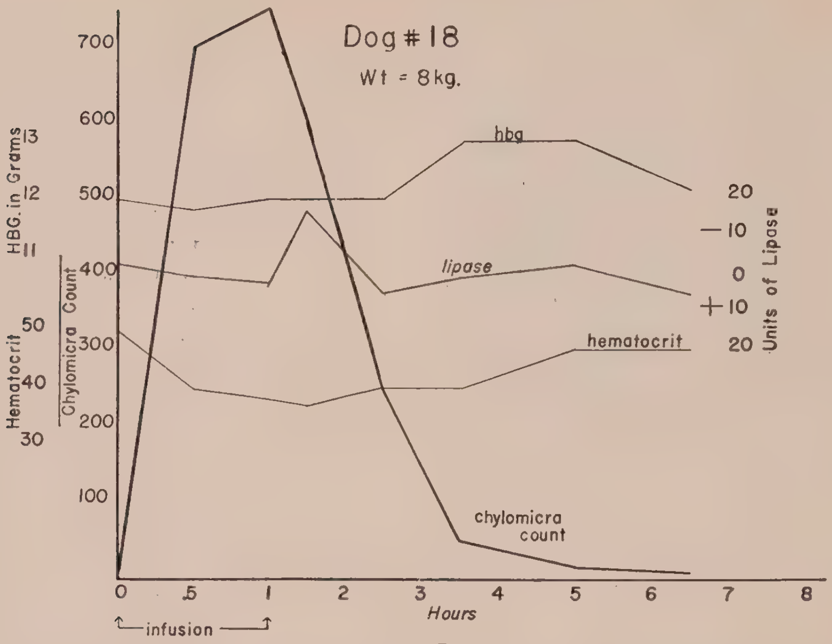


FIG. 2.

Section through the liver of the same dog. Liver shows areas of focal accumulations of fat still to be metabolized. The liver architecture is normal.

One dog received 500 ml of the emulsion within 90 minutes, the other received an equal volume over a period of 300 minutes. Both dogs tolerated the emulsion well. Plasma samples obtained during the rapid infusion were milky white in color and the emulsion could be readily detected. On nephelometric examination there was a marked increase in the suspension concentration of such samples over that of the initial plasma sample. In the former samples a "cream layer" was obtained on standing in a narrow tube or after high speed centrifugation. All plasma samples obtained during the slow infusion were clear in color and showed little turbidity as determined by the nephelometer. The rate of disappearance of the emulsion from the blood as determined by the total serum lipids and the chylomicra count differed from that of the physical disappearance of the emulsion from the plasma. Depending on the rate of infusion there was a progressive rise in the chylomicra count and the total serum lipids and within 4 hours after completion of the injection both latter values returned to the level of their fasting state in the blood.

Group II. Four dogs in this group received daily infusions of the 5% combined emulsion for a 30 day period. They were allowed in addition a standard kennel diet. Dog No. 8 was injected with 150 ml of the emulsion, dog No. 9 with 250 ml, dog No. 10 with 400 ml and dog No. 11 with 500 ml of the emulsion. The number of injected calories ranged from 127 to 425 calories daily. Dog No. 8 received a minimum of 225 g of injected fat while dog No. 11 received the maximum of 750 g of fat during the study period. Two other dogs in this group No. 12 and No. 13 were infused daily with 500 ml of the 10% combined emulsion for a period of 14 days receiving also a total of 750 g of fat. In the dogs infused with large amounts of fat there was a corresponding decrease in appetite for the kennel diet. On many occasions the dogs refused the kennel diet entirely and accepted water only which was taken in large quantities. The dogs were always alert and energetic and never offered resistance throughout the infusion phase. During the injection there was a marked elevation in the chylomicra count in the blood followed by a fall to the pre-infusion level within four hours of completion of the infusion. Lipase activity determinations run concurrently with the latter revealed a significant increase in enzymatic activity in the blood. Red blood counts and



GRAPH I.
Typical graph of the chylomicra count, hemoglobin, lipase activity and hematocrit obtained in Dog No. 18 (Group III) on the 10th study day during the infusion and post-infusion phases of an injection of 500 ml of the 10% combined emulsion.

hemoglobin determinations made at regular intervals during the 30 day period showed no signs of hemolytic anemia. The weight gain in the animals varied from 0.8 kg to 1.6 kg. The largest weight gain occurred in dogs No. 8 and No. 9 who received the smallest volume of emulsion and ate more of the kennel diet than the others of the group. Examination of the tissues revealed the liver cells to contain intra-cellular fat with an occasional focal aggregation of lymphocytes. No true granulomata were observed in the lung tissue. The architecture of the kidney and spleen was normal.

Group III. All the dogs of this group were maintained exclusively on daily infusions of 500 ml of the 10% combined emulsion for a period of 14 days. The total amount of fat injected was 750 g. The dogs were allowed water ad libitum, received orally multiple high potency vitamins and were rewarded after each infusion with 2 rye-krisp biscuits. Chylomicra counts showed the characteristic tendency to increase during the infusion and for a short period thereafter. Lipase activity increased in the blood during the infusion. The specific gravity of the blood and the plasma proteins stayed approximately stationary during the experimental period. Hemoglobin determinations and the red cell counts showed little variations from the original and secondary anemia did not develop. In this group dogs No. 14, 17 and 18 lost one-half to one kilogram in weight while dogs No. 15, 16 and 19 maintained their original weight with slight variations. Tissue studies in this group were also negative.

*Comments.* The utilization of intravenous fat emulsion for growth and energy has been effectively proved in dogs by many workers.[5,6] The present experiments have demonstrated that the combined emulsion containing 5 or 10% fat may be used intravenously both as a supplement to diet or as the sole source of

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[5] McKibbin, J. M., Ferry, R. M., and Stare, F. J., *J. Clin. Invest.*, 1946, **25**, 679.

[6] McKibbin, J. M., Hegsted, D. M., and Stare, F. J., *Fed. Proc.*, 143, **2**, 9.

provision of the minimal daily requirements of the animal. Although mild to serious anemias have been reported to develop as a result of the infusions of the fat emulsions[7,8] the latter complication failed to occur in the present investigation. This is believed to be due to the fact that phosphatide stabilizers were not used in the preparation of our emulsion. The disappearance of fat from the circulating blood determined either by the chylomicra count or by total serum lipids was interpreted to indicate that lipolysis commenced in the blood, the process being initiated by serum pancreatic lipase. Evidence to support the latter view was based on the increase of lipase activity of the blood and substantiated further by the report of Meng and Freeman[9] that the fatty acids increased during the infusion of a fat emulsion. The assimilation of preformed fat infused intravenously may therefore be conceived as a possible biochemical function whereby the neutral fat ultimately reached the liver for catabolism. An analysis of the slides revealed that in the acute experiments after an infusion all the organs were richly laden with fat. In the lung the fat did not permeate into the alveolar spaces but was contained in the capillaries. In the chronic experiments of Group II and Group III, the liver and the spleen were the only organs which contained evidences of fat.

*Summary.* (1) A combined emulsion of fat protein and glucose was infused intravenously in a series of 19 dogs.

(2). Toxic reactions such as secondary anemia and degenerative pathology in vital organs failed to develop after prolonged injections of the combined emulsion.

(3). An attempt was made to correlate the associated laboratory tests with the biochemical functions of hydrolysis and assimilation of fat.

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[7] Johnson, V., Longini, J., and Freeman, L. W., *Science*, 1943, **97**, 400.

[8] Dunham, L. J., and Brunschwig, A., *Arch. Surg.*, 1944, **48**, 395.

[9] Meng, H. C., and Freeman, S., *J. Lab. and Clin. Med.*, 1948, **33**, 689.

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### 16731

## Effect of Alloxan Diabetes on Hyaluronidase Level of the Rat Testis.*

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Although hyaluronidase, an enzyme obtained from mammalian sperm and testes, can disperse follicle cells of ovulated mammalian ova, a normal role of this enzyme in reproduction remains to be established.[1,2] In line with the main problem of determining this role it was desired to learn if a physiological disturbance of the metabolism of the animal, without the destruction of the germinal elements, could alter the level of hyaluronidase within the testis. Experimental diabetes, therefore, was induced in male rats and after one month the amount of hyaluronidase was measured in the control and diabetic rat testes.

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* Aided by a grant from the Sage Fund of the Cornell University Trustee-Faculty Committee on Research, administered by Dr. S. L. Leonard.

[1] Chang, M. C., Proc. Soc. Exp. Biol. and Med., 1947, **66**, 51.

[2] Leonard, S. L., Perlman, P. L., and Kurzrok, R., Proc. Soc. Exp. Biol. and Med., 1947, **66**, 517.

TABLE I.
Hyaluronidase Concentration in Normal and Diabetic Rat Testis. (Turbidimetric Method).

| Testes | Normal<br>No. of rats | TRU/g* | Diabetic<br>No. of rats | TRU/g | Significance of Diff. of means |
|---|---|---|---|---|---|
| Chilled, homogenate | 10 | 10.1 ± 1.5† | 10 | 10.4 ± 2.8 | P = .90 |
| Incubated, homogenate | 9 | 46.6 ± 6.6 | 9 | 52.2±17.4 | P = .75 |
| + Sem. ves. incubated | 5 | 68.1 ± 3.8 | 5 | 68.6 ± 4.3 | P = .90 |

* TRU: turbidity reducing unit.
† Standard deviation.

TABLE II.
Hyaluronidase Concentration in Normal and Diabetic Rat Testes. (Rat-Ova Test).

| Testes | Normal<br>No. of rats | Time to denude ova (min) | Diabetic<br>No. of rats | Time to denude ova (min) | Significance of Diff. of means |
|---|---|---|---|---|---|
| Chilled, homogenate | 5 | 46 ± 8.2* | 5 | 66 ± 15.2 | P = .30 |
| Incubated, homogenate | 5 | 30 ± 14.6 | 5 | 24 ± 12.7 | P = .75 |
| + Sem. ves. incubated | 5 | 35 ± 9.3 | 5 | 46 ± 10.8 | P = .65 |

* Standard deviation.

*Methods.* Adult male rats were made diabetic by injecting alloxan intraperitoneally (175 mg/kg body weight). Qualitative determinations for glycosuria in the alloxan treated rats were made. In some cases quantitative blood sugar determinations[3] were performed because it was thought that hyaluronidase concentration might vary with the degree of hyperglycemia. Body weights were obtained before and after alloxan administration; after 30 days the testes of each rat were removed and assayed for hyaluronidase.

One of the two assay procedures used was the turbidimetric method as previously described for chilled testis homogenates.[4] Since it has been reported that incubation of the testis homogenate increases the yield of the enzyme,[5] a series of experiments was performed with the additional step of incubation. The amount of enzyme is reported as "Turbidity reducing units" (T.R.U.). The other assay procedure, the rat-ova test, was modified from the original procedure[6] in that comparisons were made of the time required for denudation of rat ova of their follicle cells. Saline extracts of testes from normal and diabetic rats were prepared in concentrations which were the same for both the experimental and control testes in each assay.

An additional test was included in this study. Comparisons were made in the yields of enzyme produced in the experimental and control groups when macerated seminal vesicles were mixed with the testis homogenates before extracting. It was shown that increased yields of enzyme could be obtained when the seminal vesicles were added.[7]

*Results.* The hyaluronidase concentration of the testes of normal and diabetic rats are presented in Tables I and II. In no case was the enzyme concentration in the testes of the experimental and control rats significantly different. When the testis homogenate was incubated before extraction an increase in enzyme concentration was obtained in both groups. In the rat ova test, the amount of extract in gram-equivalents of testis material was purposely lowered when the testis homogenate was incubated, otherwise the reaction would have proceeded so rapidly that an accurate comparison of the time of denudation of the ova would be impossible. With the chilled testis homogenates equivalents of .05 g of tissue were employed and with the incubated testes .01 g of tissue.

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[3] Sumner, J. B., *J. Biol. Chem.*, 1925, **65**, 393.

[4] Leonard, S. L., Perlman, P. L., and Kurzrok, R., *Endocrinology*, 1946, **39**, 261.

[5] Perlman, P. L., Leonard, S. L., and Kurzrok, R., *Endocrinology*, 1948, **42**, 26.

[6] Leonard, S. L., and Kurzrok, R., *Endocrinology*, 1946, **39**, 85.

[7] Leonard, S. L., Perlman, P. L., and Kurzrok, R., *Endocrinology*, 1947, **40**, 199.

In the experiments where the seminal vesicle material augmented the yield of hyaluronidase no significant difference was observed between the amount of enzyme obtained from normal and diabetic animals (Tables I and II). The amount of enzyme present in the seminal vesicle was practically negligible. In the rat-ova test .005 g of tissue equivalent were required to denude the ova in the time indicated.

Although the diabetic rats exhibited hyperglycemia during the period of the experiments no correlation was observed between blood sugar levels and the enzyme concentrations of the testes. No histological changes were observed in the testes of the experimental rats, and there was no altered breeding performance as measured by the size of the litters sired.

In contrast to these results destruction of the germinal epithelium by hypophysectomy or cryptorchidism is followed by a marked decrease in ryaluronidase levels.[8] Whether the enzyme level of the germinal cells of the testes can be altered by physiological changes which do not destroy these germ cells remains to be shown.

*Summary.* 1. Alloxan diabetes in the male rat had no effect on the hyaluronidase concentration of the testes.

2. Augmentation of the yield of extractable hyaluronidase from testis homogenates by seminal vesicle homogenates was confirmed. No difference in augmentation was noted between the normal and diabetic rat testes.

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[8] Leonard, S. L., Perlman, P. L., and Kurzrok, R., *Endocrinology*, 1948, **42**, 176.

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